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5

Radioimmunoassay of Prostaglandins and Thromboxanes

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Radioimmunoassay of prostaglandins was first introduced in 1970 by Levine and Van Vunakis with assays developed for PGE_1 and $PGF_{2\alpha}$. Since then the literature in the field has been rapidly expanding, and a large number of radioimmunoassays for prostaglandins, prostaglandin metabolites, prostaglandin analogs, and thromboxanes have been published. Because of space limitation, the authors of the present chapter did not aim at writing a comprehensive review where all methods are discussed. Rather, we present general guidelines for scientists who want to develop a prostaglandin radioimmunoassay and discuss the more important aspects in the experimental design of a study and the pitfalls in the interpretation of data.

INTRODUCTION

Radioimmunoassay (RIA) is based on the competition between labeled and unlabeled molecules of a particular compound for binding sites of an antibody directed against the same substance. The amount of labeled compound is known and constant for all the tubes in an experiment, whereas the amount of

unlabeled substance is known and varied (standard tubes) or unknown (sample tubes). In each experiment there must also be tubes that indicate "zero binding" (no antibody present) and "maximal binding" (no unlabeled substance added apart from the few unlabeled molecules that may be present in the labeled preparation).

In the presence of few unlabeled molecules, a large proportion of the labeled ones are bound by the antibodies. When larger amounts of unlabeled substance are present, the radioactive molecules are displaced from the binding sites and the radioactivity of the unbound fraction becomes greater (Fig. 1). The free and the antibody-bound fractions are separated, and the radioactivity of either or both determined. The exact amount of compound in a sample tube is then obtained by comparison with the standard tubes.

Several comprehensive books and reviews on this technique have been written (e.g., Kirkham and Hunter, 1971; Berson and Yalow, 1973; Spector, 1973; Sönksen, 1974; *Radioimmunoassay and Related Procedures in Medicine*, 1974; *Standardization of Radioimmunoassay Procedures*, 1974).

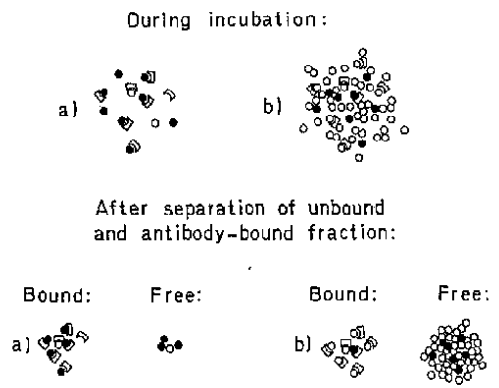

FIG. 1. Basic principles of RIA. (M) Antibody. (O) Unlabeled molecule. (●) Labeled molecule. The incubation is carried out in the presence of a (a) small and (b) large amount of unlabeled substance. The amount of radioactivity is the same in both incubations. The radioactivity of the free fraction in this experiment more than doubled in case b compared to case a.

RIA has certain advantages in comparison with other quantitative methods. First, it is possible to reach a high sensitivity, and the limit of detection may be as low as 10 fmoles of prostaglandin per sample. Precision and accuracy frequently compare favorably with other methods. The specificity may under certain circumstances be considerable. The method is comparatively rapid: an assay of, for example, 100 samples can be completed within 1 to 2 days, including radioactivity measurements and processing of data. Provided the samples are not pretreated extensively prior to assay (extraction, chromatography, and so forth), RIA also has a higher sample capacity than most other quantitative methods in this field. Finally, the only major equipment that is indispensable is the radioactivity counter; otherwise, this type of assay can be acceptably carried out without complicated and expensive laboratory equipment.

However, there are also a number of drawbacks. First, RIA is not an entirely specific method under all circumstances. The specificity of the *antibody* is generally very high, but this does not imply that the data obtained are completely reliable. (This problem is discussed in more detail later in the chapter.) The second disadvantage is that, contrary to popular belief, the method is a very expensive one. To reach a high degree of reproducibility, it is necessary to use disposable glassware and other disposable utensils as much as possible. Commercial antisera are generally expensive, particularly the so-called second antibodies, which are employed in the double-antibody techniques. This last-mentioned type of method also requires unusually large volumes of sera, since the anti-γ-globulin sera generally are of low titer. If the scientist prefers to raise his own antisera, the cost becomes almost negligible; however, this requires facilities for animal housing in the laboratory. The labeled ligands are very expensive, if they can be commercially obtained at all. Even if they are synthesized in the scientist's own laboratory, the labeled precursors are generally of high cost. Furthermore, large amounts

of scintillation fluid are required in case a β-emitting type of label is used, as well as disposable counting vials. Finally, the processing of data is, without comparison, best performed with the aid of a computer, which adds considerable extra cost to the method. In our laboratory, we calculate an overall material cost of \$1 to \$3 per tube, but we always employ our own antisera.

Before the development of a quantitative method for the determination of prostaglandins or related compounds, it is necessary to consider certain aspects. The general approach to this problem was discussed in the introductory chapter of this book (page 1). Briefly, it is advisable to start with the choice of biological material suitable for the study; for example, measure prostaglandin levels in plasma in order to study rapid fluctuations in prostaglandin release, in urine when this is not necessary or impossible, or in some special fluid or other material in other cases. After this decision has been made, the suitable compound is selected for the study: a prostaglandin, a prostaglandin metabolite, a thromboxane, and so forth (Fig. 2).

In case the scientist prefers to employ RIA for his determinations, the next step must be to consider whether the selected compound is suitable for such a method. This requires among other things a considerable stability, both chemically (in aqueous solution; during the coupling procedure) and metabolically (metabolism may take place even in the conjugate). The need for chemical stability rules out compounds like thromboxane A (TXA) and the endoperoxides. However, it is by no means impossible to use RIA for the measurement of these compounds; they cannot be assayed as such but must first be converted to stable derivatives. Figure 3 shows some possibilities for the conversion of unstable compounds into other products more suitable for assay. TXA_2, for example, may be converted into mono-O-methyl TXB_2 by excess methanol. The endoperoxides PGG_2 and PGH_2 can easily be reduced to $PGF_{2\alpha}$.

Prostaglandins of the E type are also known to give rise to problems in this field because they are easily dehydrated during

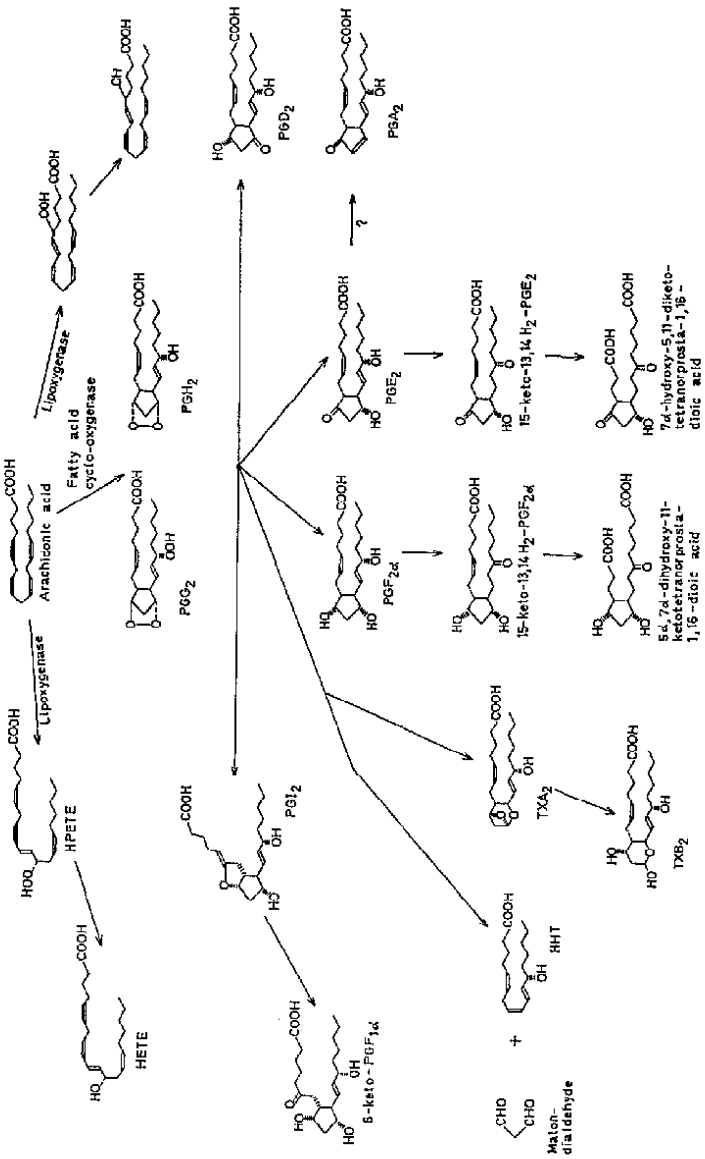

FIG. 2. Metabolism of arachidonic acid in the human.

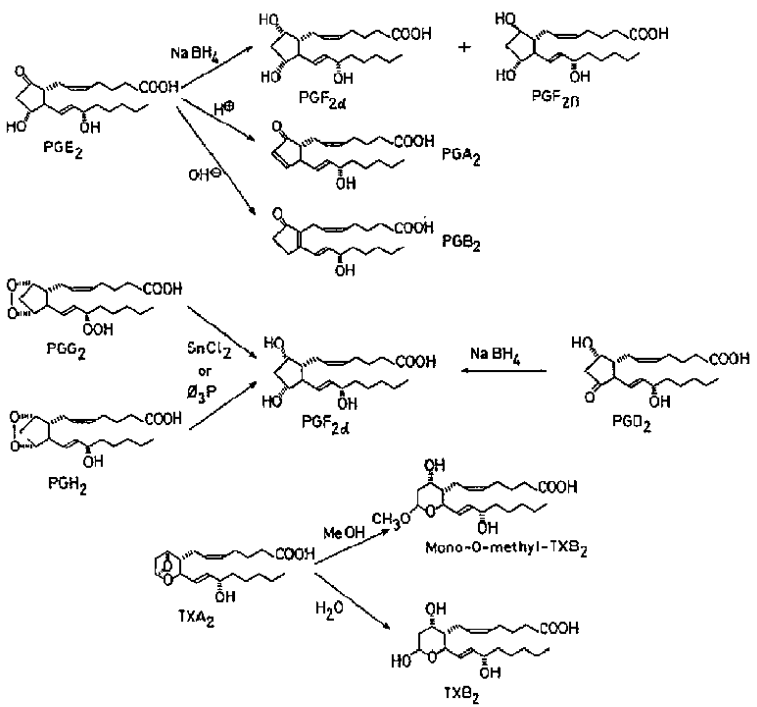

FIG. 3. Chemical conversion of some prostaglandins and thromboxanes into more stable derivatives suitable for radioimmunological measurement.

the coupling procedure to form PGA or PGB compounds. A similar problem is likely to pertain also for PGD compounds. Thus one approach to the development of radioimmunological methods for prostaglandins E and D is first to convert these substances into other prostaglandins that are not dehydrated during the coupling conditions, and develop the assay for those instead (PGE to F_α and F_β, or to A or B; PGD to F_α) (Fig. 3).

One last aspect that should be kept in mind is that it is advantageous to choose a compound with many strong antigenic determinants if success in raising a RIA is to be expected. The

prostaglandins and the thromboxanes are evidently very good antigens, and the resulting antisera seem to be very specific for these compounds and will recognize, for example, the respective ring structures as well.

On the other hand, a compound such as HHT or HETE (Fig. 2) cannot be expected to give rise to good antisera. The structures of these compounds are far from "specific." If these compounds are to be monitored, RIA is not recommended.

DEVELOPMENT OF THE RADIOIMMUNOASSAY

Materials and Equipment

The major materials for the assay include:

1. The substance, a few milligrams for preparation of the conjugate and for use as standard.
2. Labeled ligand ("tracer").
3. Reagents for separation of bound and free fractions.

The equipment needed for the assay includes the following:

1. Animals for immunization.
2. Pipetting equipment.
3. Vortex mixer.
4. Disposable glassware and pipette tips.
5. Refrigerated centrifuge with high sample capacity.
6. Radioactivity counter, generally a scintillation counter.
(7. Computer, if available.)

Other equipment includes facilities for chromatography, for example, thin-layer chromatography and thin-layer scanner for checking the purity of the labeled ligand; silicic acid or reversed phase partition chromatography for purification of labeled ligand, etc., and, if necessary, facilities for extraction of samples and chromatography of extracts on microcolumns.

Several commercial antibodies for prostaglandins are currently available. Second antibody (directed against the γ-globulin of

the species employed for the raising of the first antibody) is also commercially available, being produced in sheep or goat. Some manufacturers have either complete kits (which contain everything needed for the RIA) or sets (which often lack the radiolabeled ligand).

Antibodies, or sometimes complete kits, for primary prostaglandins can be purchased from the following sources.
For the primary prostaglandins:

anti-PGE:	Calbiochem, Clinical Assays
anti-PGE_1:	Sigma Chemical Company, Institut Pasteur
anti-PGE_2:	Sigma, Miles Laboratories Ltd., Institut Pasteur
anti-PGF:	Calbiochem, Institut Pasteur
anti-$PGF_{1\alpha}$:	Calbiochem, Clinical Assays, Institut Pasteur
anti-$PGF_{2\alpha}$:	Sigma, Miles, New England Nuclear, Clinical Assays

For the A and B series:

anti-PGA and B:	Calbiochem
anti-PGA, B, and E:	Calbiochem
anti-PGA_1:	Sigma, Institut Pasteur
anti-PGB_1:	Sigma, Institut Pasteur

For prostaglandin metabolites:

anti-15-keto-13,14-dihydro-$PGF_{2\alpha}$: Clinical Assays

(Sigma Chemical Company, St. Louis, Mo.; Calbiochem, Lucerne, Switzerland and La Jolla, Calif.; Miles Laboratories, Stoke Poges, Slough, England and Elkhart, Ind.; Clinical Assays, Cambridge, Mass.; New England Nuclear, Dreieichenhain, West Germany and Boston, Mass.; Institut Pasteur, Paris, France).

The quality of these kits or antibodies varies considerably between manufacturers and also between batches from the same company. If the scientist prefers to use commercial preparations, it is important to check the reagents prior to use: the purity of the labeled ligand, the titer of the antiserum, and so on, and further to scrutinize carefully the recommended procedure. It

is often seen that the conditions described in the manual are far from optimal (cf. Drewes, 1974).

SUBSTANCE FOR PREPARATION OF CONJUGATE AND FOR USE AS STANDARD

For Conjugation

The amount of prostaglandin necessary for the production of a good antiserum is very small; generally, 1 to 10 μmoles is sufficient. Many prostaglandins can be obtained (e.g., from The Upjohn Company, Kalamazoo, Mich.; ONO Pharmaceuticals Co., Osaka, Japan; Sigma Chemical Company; Calbiochem; and ICN Pharmaceuticals, Cleveland, Ohio). Most scientists seem to have obtained their material from The Upjohn Company. These compounds include the common prostaglandins, a few metabolites (some 15-keto, 15-keto-13,14-dihydro, and 13,14-dihydro compounds), and certain prostaglandin analogs (e.g., 15-methyl prostaglandins).

The common urinary metabolites and the thromboxanes are not yet available and must be prepared in the scientist's own laboratory. The total synthesis of prostaglandins and related compounds is difficult and perhaps beyond the capacity of most laboratories, but a partial synthesis of the desired compound from a related substance is often very simple and could be carried out by anyone. Biosynthetic preparations, using either *in vitro* or *in vivo* systems, may be quite easy to perform. The 15-keto-13,14-dihydro metabolites of the prostaglandins are best prepared biosynthetically *in vitro* using either commercially available preparations of the enzymes 15-hydroxy prostanoate dehydrogenase and Δ^{13} reductase (ICN Pharmaceuticals) or simply by an incubation with the high-speed supernatant from an organ with a high capacity for this conversion, such as the lung or kidney from many species (Samuelsson et al., 1975). Dinor and tetranor metabolites can be prepared from the parent compound using the β-oxidizing system of rat or guinea pig liver

mitochondria (Hamberg, 1968). It is convenient to include a small amount of radiolabeled precursor in the preparation of all these compounds. If this is done, purification of the product is greatly facilitated, estimation of the formed amount of product becomes considerably more certain, and it is possible to measure the molar ratio in the later prepared prostaglandin–carrier conjugate (see below). However, if the same preparation is also to be used as standard, the amount of radioactivity included must be sufficiently low so as not to disturb the later radioactivity measurements in the RIA. A suitable alternative is to employ a different label than the one used for the labeled ligand (e.g., ^{14}C-labeled standards of low specific activity in an assay where the tracer is labeled with ^{3}H and of high specific activity).

Some metabolites must be prepared *in vivo*. Total syntheses for the tetranor dioic acids, which constitute the major degradation products of $PGF_{2\alpha}$ and PGE_2 in the human, have been developed (e.g., Lin, 1976), but for most laboratories it is easier to prepare the main urinary metabolites of PGE_2 and $PGF_{2\alpha}$ *in vivo*. 5α,7α-Dihydroxy-11-ketotetranorprosta-1,16-dioic acid is found in the urine of several species after injection of $PGF_{2\alpha}$, for example, the human (Granström and Samuelsson, 1971*a*), the rabbit (Svanborg and Bygdeman, 1972), and the rat (Gréen, 1971). The compound is a major metabolite only in the human species, representing 20 to 25% of the given amount of $PGF_{2\alpha}$. If the scientist can collaborate with a gynecological department where $PGF_{2\alpha}$ is employed as abortifacient, amounts of the metabolite sufficient for the production of an antiserum can be obtained from a single patient. Small amounts of the labeled compound must be added for purifying the substance from urine (see below). The compound is isolated as the methyl ester by a few chromatographic steps (Granström and Samuelsson, 1971*a*), and since this compound occurs as a δ-lactone as well as in the open form (Fig. 7, below), both forms should be collected and hydrolyzed together prior to coupling to the protein carrier.

Other tetranor prostaglandins, which occur as urinary metabolites in other species, do not have to be prepared *in vivo*. This is the case, for instance, with the major metabolites of PGE_2 and

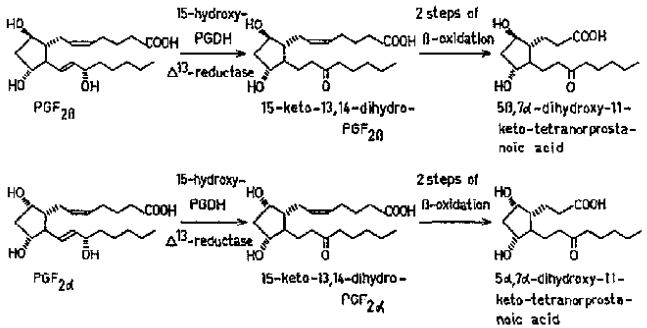

FIG. 4. *In vitro* preparation of the major urinary metabolites of PGE_2 and $PGF_{2\alpha}$ in the guinea pig.

$PGF_{2\alpha}$ in the guinea pig, *viz.*, 5β,7α-dihydroxy-11-ketotetranorprostanoic acid and 5α,7α-dihydroxy-11-ketotetranorprostanoic acid. These can easily be prepared *in vitro* with good yield (Fig. 4) (Hamberg and Samuelsson, 1969).

The preparation of thromboxanes or thromboxane derivatives is best carried out *in vitro* using platelets or lung tissue and the proper fatty acid as precursor (Granström et al., 1976*a*,*b*). Arachidonic acid, incubated with a platelet suspension, is rapidly converted to TXA_2 (Fig. 2); if the incubation is interrupted after about 30 sec with large volumes of methanol, the two epimers of mono-O-methyl TXB_2 can be isolated among the major products (Fig. 3) (Hamberg et al., 1975). On the other hand if the incubation is continued for 10 min or more prior to interruption, the unstable TXA_2 will instead have been converted into TXB_2 in the aqueous medium (Fig. 3).

For Standard

For any compound that has to be prepared biosynthetically, it is advisable to search the literature to find the best species

and/or the best organ for the desired conversion. For biosynthesis of larger amounts (a few milligrams) for use in the preparation of the antigenic conjugate, it is not necessary to know the exact quantity of the formed product with any higher degree of certainty. Thus when crude enzyme preparations are employed for the biosynthesis, or when *in vivo* preparations are carried out, it is hardly necessary to suppress the endogenous formation of the compound; this contribution is of minor importance.

However, when making the preparation that is to be used as standard, the situation is different. It is now necessary to know the exact amounts formed, and thus any endogenous production of the compound must be inhibited, since this contribution is often unknown and variable. This problem does not arise when purified enzymes are used for the biosynthetic conversion, for example, when carrying out β-oxidation with washed mitochondria or preparing 15-keto prostaglandins with a purified prostaglandin dehydrogenase. However, when crude enzyme preparations are employed, the endogenous contribution becomes considerable unless the rapidly deep-frozen organ is ground directly into and homogenized in a buffer containing sufficient amounts of prostaglandin synthetase inhibitors (Gréen et al., 1973). The *in vivo* preparation of standards is even more complicated. One method for the preparation of small and exactly known amounts of, for example, 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid, is to give an intravenous infusion of [9β-^{3}H]-labeled $PGF_{2\alpha}$ of an exactly known and sufficiently low specific activity to a human volunteer, or even one of the other species that degrade $PGF_{2\alpha}$ to this dioic acid. The subject that receives this infusion must be pretreated with, for example, indomethacin for several days, and it is recommended that urine be collected during a period immediately prior to the infusion for a later determination of the actual basal metabolite production under these conditions. It is also advisable to collect the urine containing the labeled products for as short a time as possible in order to minimize further the endogenous contribution. The substance is then purified from the urine, and the amount obtained can be calculated with a fairly high degree of certainty (Granström and Kindahl, 1976*b*).

Standard dilutions. The stock solution of the standard is preferably made in a nonaqueous solvent, particularly for the PGE compounds. The working solutions are prepared in buffer in suitable dilutions. A geometrical dilution is recommended (see below).

These preparations must be checked regularly for purity, as the RIA results obviously will be misleading if any degradation of the standard occurs. Prostaglandins of the E-type are unstable even in aqueous solutions around neutral pH, and may be quite extensively degraded to PGA and PGB compounds under these conditions within only a few weeks. This problem is rarely recognized, and in publications dealing with RIA methods for PGE compounds information is seldom given about how frequently the standard dilutions were prepared, thus casting some doubt on the reported PGE values, cross reactions with PGE compounds, and so forth.

Use of Alternative Compounds

A common problem is that a scientist considers the difficulties in obtaining a certain compound too great and contents himself with a related substance instead. For instance, several laboratories have in the past developed RIAs for 15-keto prostaglandins instead of the 15-keto-13,14-dihydro compounds for use in peripheral plasma measurements (e.g., Levine and Gutierrez-Cernosek, 1972). The circulating amounts of the 15-keto compounds are very small in comparison with those of the major metabolites in the circulation. Another example is the development of RIAs for prostaglandins of the "1" series, which generally are of less importance than those of the "2" series (Jaffe et al., 1971; Levine et al., 1971; Stylos et al., 1973).

In some cases the intention is to take advantage of the cross reaction that can be expected to be exhibited by the resulting

antiserum, *viz.*, the cross reaction with a more important compound that is known to occur in several-fold higher concentration in a particular system. This approach, however, requires that the major compound (the one really intended to be measured) is used for both standards and tracer if the antiserum is raised against the "wrong" (minor) compound. The resulting measurements in such a case are likely to reflect the levels of the major compound; however, the working titer of the antiserum can be expected to be low and the assay probably insensitive.

Sometimes it is seen that the "wrong" compound is employed not only for production of the antiserum but for labeled ligand and standard as well. In these cases the resulting measured levels cannot be trusted; the main metabolites or compounds are likely to cross react in the assay, and it cannot be assumed that the concentrations of major and minor compounds always vary in a parallel fashion.

We do not advise using any of these approaches. As the amount of work and time that must be spent in the development of a RIA is considerable, it is definitely advisable to spend some more time and effort at the first stage to find the proper compound instead of saving a few weeks and ending up with a RIA with very limited value.

However, the opposite situation may in fact be advantageous, that is, the use of an antiserum raised against a major compound and a heterologous labeled ligand. This may increase the sensitivity of the assay somewhat, but the appropriate compound must then be used for standards.

PREPARATION OF CONJUGATE

Nature of Carrier

Substances of low molecular weight like prostaglandins are not antigenic in themselves but must be coupled to a larger molecule to be able to evoke an immune response. The carrier molecule may be a protein (e.g., albumin) or a polypeptide (e.g., polylysine). Among the proteins, bovine serum albumin (BSA) has been most commonly employed, but others (e.g., γ-globulins or thyroglobulin) are sometimes used (Jubiz et al., 1972; Stylos et al., 1974; Raz et al., 1975; Christensen and Leyssac, 1976). Conjugates prepared with polylysine must be further coupled to, for example, keyhole limpet hemocyanin prior to injection into the animal (Levine et al., 1971; Attallah and Lee, 1973). Some also use a second carrier: the PG–protein or PG–polypeptide carrier is absorbed on *Pneumococcus* strain cells (Stylos and Rivetz, 1972; Raz and Stylos, 1973). This second carrier is employed to prevent the possible *in vivo* conversion of prostaglandins, in this case of PGA_1 to PGC_1, which has been thought to be the explanation for the high cross reactivity of certain antisera with other prostaglandins and metabolites.

Coupling Methods

When preparing a hapten–protein conjugate for immunization, it is necessary to select the site of coupling carefully. Antibodies combine with the available surface of the hapten and recognize these parts of the molecule well, whereas the structures close to and at the coupling site seem to be of minor importance. The prostaglandin molecule offers a suitable coupling site, the carboxyl group, which has two advantages. First, relatively mild methods can be used in coupling a carboxyl group to amino groups on the protein molecule; and second, the carboxyl group and the whole carboxyl side chain are generally not particularly interesting parts of the molecule. Other possibilities, for instance, would be to use keto groups or hydroxyl groups in the ring and at C-15; however, these parts of the molecules are more important than the carboxyl side chain in this respect, and they must be well recognized by the antibody. This is particularly the case with the ring structures, which have repeatedly been proved to be strong antigenic determinants. Thus the coupling should take place as far from these structures as possible.

The only drawback of using the carboxyl group for the con-

jugation is that the Δ^5 double bond in prostaglandins of the "2" series is sometimes not well recognized, and cross reactions with those of the "1" series are often high, even 100%. This need not always be a drawback, however, as it makes possible the use of a heterologous tracer (see below).

Several coupling methods exist that are suitable for conjugating carboxyl groups to amino groups. One possibility is the use of the isobutyl chloroformate method (e.g., Kirton et al., 1972). The most commonly employed method, however, is the use of a water-soluble carbodiimide (CDI) as a coupling reagent (Caldwell et al., 1971). Figure 5 illustrates such a procedure. The method is simple and rapid, and the coupling may be carried out as follows: 10 mg of, for example, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ is dissolved in 2 ml dimethyl formamide. In a second vessel 40 mg BSA is dissolved in 2 ml water, and the pH is adjusted to 5.0 to 5.2. With constant stirring, 20 mg 1-ethyl-3(3-dimethylaminopropyl)-CDI is added while maintaining the pH at 5.0 to 5.2. The prostaglandin solution is added

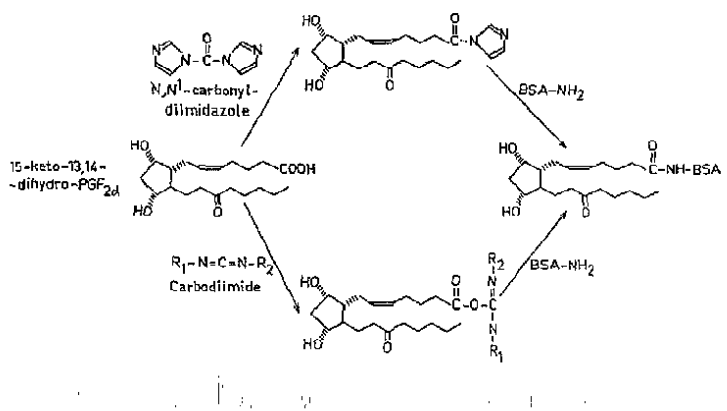

FIG. 5. Preparation of the PG–BSA conjugate using either N,N′-carbonyldiimidazole or a water-soluble carbodiimide as coupling reagent.

dropwise with constant stirring to the CDI vessel, and after 2 hr another 5 mg CDI is added. The mixture is kept at room temperature for 24 hr with stirring and then extensively dialyzed for several days against water. The contents of the dialysis bag are subsequently lyophilized for later injection into animals (Caldwell et al., 1971).

This method is simple but has been reported to dehydrate prostaglandins of the E type extensively to A or B compounds. Recently a different, milder method was described, which was reported not to dehydrate PGEs (Axen, 1974). In this procedure N,N′-carbonyldiimidazole was employed as the coupling reagent. A convenient procedure is the following (Fig. 5): The prostaglandin, for example, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (10 mg), is dissolved in 2 ml dry dimethylformamide and kept under N_2 at room temperature with stirring for 10 min. N,N′-Carbonyldiimidazole (5 mg) is added, and the reaction mixture is kept for a further 20 min under N_2 with constant stirring. This mixture is then added dropwise to a solution of 40 mg BSA in 3 ml water; after 5 hr at room temperature, 5 ml of a dimethylformamide:water mixture (2:3, v/v) is added and the reaction mixture dialyzed, first against dimethylformamide:water (2:3) for 24 hr and then against several changes of water for 48 hr. The contents of the dialysis bag are subsequently lyophilized.

Using either method, a certain amount of protein is denatured and found as a precipitate. This precipitate is treated by some investigators with trypsin (Stylos and Rivetz, 1972). In our experience this does not seem to be necessary. If the protein precipitates late during the procedure, it is likely to contain conjugated prostaglandin and may work well as an antigen. It may even be advantageous for the immune response to administer a particulate antigen. Thus we never remove the precipitate found in the dialysis bag but lyophilize the whole content of the bag in one vessel.

The amount of conjugate obtained in our laboratory after either of the two procedures described above has generally been divided into eight equal portions and used for two rabbits (four

injections each according to the immunization schedule below). Recently, however, we found that good antisera can be produced with considerably smaller quantities of antigen.

Estimation of Molar Ratio

As mentioned above, it is convenient to include a small, known amount of the labeled prostaglandin when preparing the conjugate. It is then possible to estimate the amount of prostaglandin that has been incorporated in the final conjugate. This estimation should not be performed until an extensive dialysis has been done.

This molar ratio, or degree of substitution, in the conjugate does not seem to be as important for the production of prostaglandin antisera as for steroids and certain other compounds. The molar ratio of PG:BSA has a theoretical maximum of 52, there being 52 free amino groups in the BSA molecule. This ratio, however, is never reached. Molar ratios generally ranging from 1.5 to 23 have been reported (Caldwell et al., 1971; Jaffe et al., 1971) using the same coupling procedure, and all conjugates have given rise to good antisera. Our own attempts to prepare conjugates have never given a degree of substitution higher than 10, yet the resulting antisera have had high titers. Perhaps the higher values reported were sometimes caused by inadequate dialysis, with some free labeled prostaglandin remaining in the dialysis bag and being counted together with the labeled conjugate. Dray et al. (1972) used the CDI method and reported a molar ratio of 40 in a BSA–$PGF_{2\alpha}$ conjugate.

Effect of Coupling Site on the Properties of Resulting Antibodies

The antisera produced later using PG–carrier conjugates prepared in either of these two ways generally exhibit similar properties; the more distant a certain structure is from the coupling site, the better it will be recognized by the antibodies. Figure 6

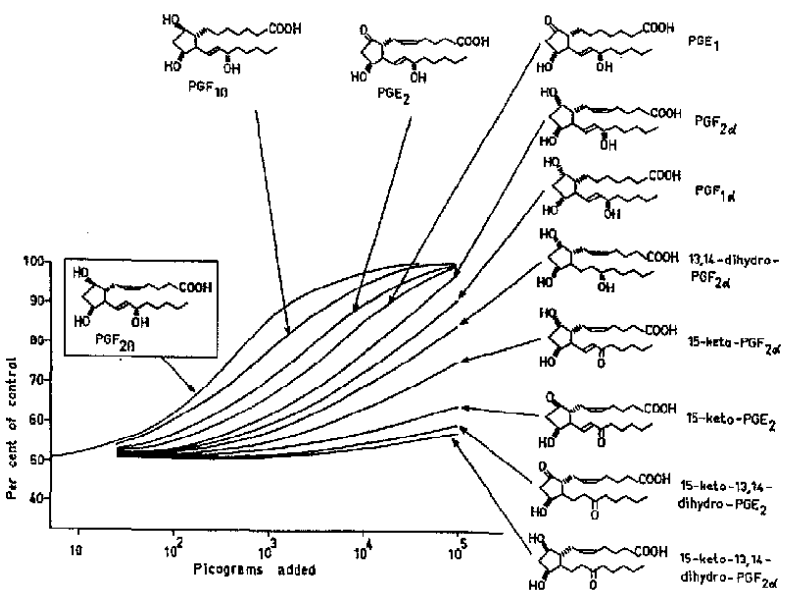

FIG. 6. Properties of an antiserum raised against a BSA conjugate with $PGF_{2\beta}$.

shows the properties of an antiserum raised against a BSA conjugate of $PGF_{2\beta}$ prepared with carbonyldiimidazole as described above. (The meaning of the cross reactivity curves is described below.) The proper compound, $PGF_{2\beta}$, is best recognized by the antibodies. If the molecule is slightly changed close to the carboxyl group, as is the case with $PGF_{1\beta}$, somewhat larger amounts of this compound are required to effect the same inhibition of the antigen–antibody binding. In spite of the structural alteration, however, the molecule is bound by the antibody; the cross reaction with $PGF_{1\beta}$ is as high as 52%. Changes in the ring are better detected by the antibodies, and higher amounts of compounds such as PGE_2 and $PGF_{2\alpha}$ are needed for the displacement of the labeled $PGF_{2\beta}$ molecules (cross reactions 22% and 5.6%, respectively). Changes in the methyl side chain,

being far from the coupling site, are even better recognized than the alterations in the ring: the cross reactions with the 15-keto-13,14-dihydro prostaglandins are less than 0.1%.

Special problems arise when working with the dioic acids that constitute the major urinary metabolites of prostaglandins in the human (Fig. 2). If the coupling procedures described above are applied as such to these compounds, no doubt the conjugation will occur randomly at either carboxyl group. If such a heterogenous antigen is injected, the resulting antiserum can be expected to be very nonspecific and contain several clones of antibodies directed at different parts of the molecule. It is thus desirable to block one of the carboxyls during the coupling procedure in order to obtain a homogenous conjugate. To date, no such method has been found for the PGE_2 metabolite 7α-hydroxy-5,11-diketotetranorprosta-1,16-dioic acid. For 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid, however, the problem can easily be solved. This compound has a pronounced tendency to form a δ-lactone, where the lactonization occurs be-

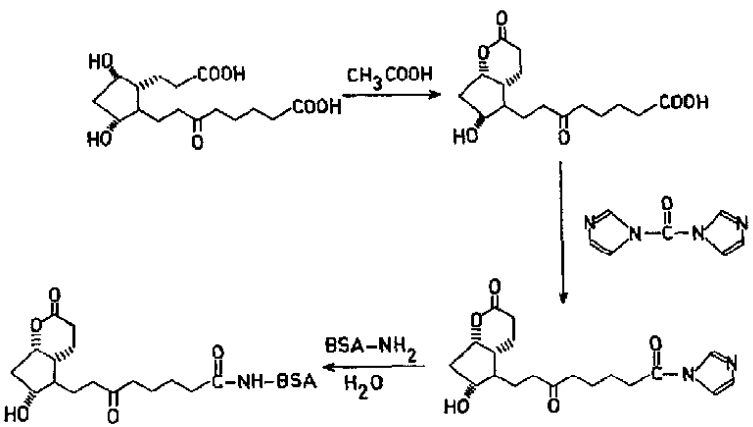

FIG. 7. Selective coupling of BSA to the ω-carboxyl group of 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid after protection of the α-carboxyl group by δ-lactone formation.

tween the carboxyl group in the original carboxyl side chain and the 5α-hydroxyl group (Fig. 7) (Granström and Samuelsson, 1971*a*). In an acidic medium such as glacial acetic acid, this equilibrium is shifted far to the lactone form. After evaporation of the acetic acid, the lactone is not likely to hydrolyze in a non-aqueous medium. Thus the coupling can be carried out in the normal way, this time by activation of the ω-carboxyl group by, for instance, N,N′-carbonyldiimidazole in dimethylformamide. This method was employed in a recently published study on the development of an RIA for the metabolite in question (Granström and Kindahl, 1976*b*). The properties of the resulting antiserum confirmed the selectivity of the conjugation: the antibodies did not seem to recognize the ω-end of the molecule at all but were very specific for the tetranor side chain. This is exactly what might be expected if the conjugation had taken place exclusively at the ω-carboxyl group (Fig. 7). The properties of this antiserum are shown and described in Fig. 8.

Two other laboratories have also reported on the raising of antisera against the main urinary metabolite of $PGF_{2\alpha}$ (Ohki et al., 1974, 1975, 1976; Cornette et al., 1975). However, when the conjugates were prepared, no special precautions were taken to convert the compound completely to the δ-lactone form. It was considered that a pH of 5.0 to 5.5, which is the normal pH during CDI coupling, would be sufficient (Ohki et al., 1974). This, however, is not the case (Granström, *unpublished;* cf. also Granström and Samuelsson, 1969). The properties of the resulting antisera were not investigated in detail; only C_{20} prostaglandins were tested for cross reactivity. Thus it is likely that these antisera contained antibodies directed at both types of the conjugate; the low titer obtained in one case (1:22, later increased to 1:1,600) (Ohki et al., 1976) may also be explained by the use of a heterogenous antigen.

Our own antiserum, which has been raised exclusively against the ω-carboxyl conjugate, is not quite homogenous either, however. As soon as the conjugate is dissolved in aqueous medium, and particularly after injection into the animal, the normal equi-

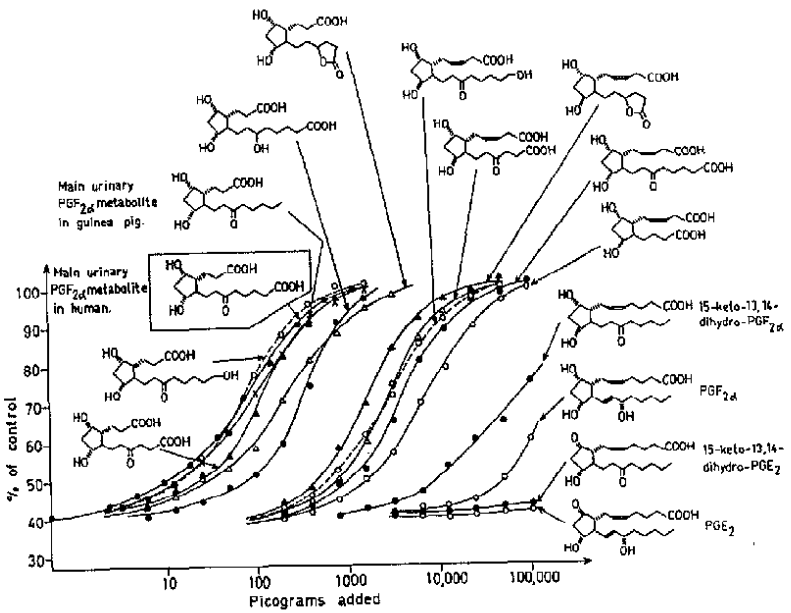

FIG. 8. Properties of an antiserum raised against a conjugate prepared as described in Fig. 7. The antibodies cross react to a minor degree with C_{20} prostaglandins and somewhat more with F compounds and compounds with the 15-keto-13,14-dihydro structure. The central cluster of displacement curves shows that the antibodies cross react to a somewhat greater degree with dinor metabolites, and that the structure at the ω-end of these compounds is of minor importance. The group of displacement curves at the left indicates that the antibodies are nonselective with respect to tetranor metabolites and cross react to a great degree with all these compounds. Again, the structure at the ω-end does not influence the affinities of the compounds to the antibodies to any greater extent. These data taken together indicate that the coupling of the hapten to the BSA molecule really took place exclusively at the ω-end (Fig. 7).

librium between the lactone form and the open form of the metabolite is no doubt reestablished (cf. Fig. 7). Thus antibodies directed at both forms are produced. However, this is no drawback, as the RIA is always carried out at a slightly alkaline pH (at least 7.4), and thus the same equilibrium is also found for

standards, unknowns, and tracer. In fact, the whole assay is a mixed assay for both the open form and the lactone form of the compound.

The coupling of the urinary $PGF_{2\alpha}$ metabolite at the C_{16} end introduces two more advantages into the assay. First, the resulting antibodies cross react extensively with other tetranor compounds, as shown in Fig. 8. It is probably seldom necessary to assay 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid exclusively; in most studies the method is used to follow changes in the $PGF_{2\alpha}$ production in the body, and thus it is no drawback that the antiserum also reacts to some extent with the other, minor $PGF_{2\alpha}$ metabolites. The second advantage is that the antiserum can be used also for other species that degrade $PGF_{2\alpha}$ to other tetranor products. For example, in the guinea pig the main urinary metabolite of $PGF_{2\alpha}$ is 5α,7α-dihydroxy-11-ketotetranorprostanoic acid, which cross reacts 87% with this antiserum; the method has in fact been successfully applied to studies in this species (Granström and Kindahl, 1976*b*). Furthermore, it is possible to use the latter compound as the labeled ligand—a great advantage, as it can be prepared with a considerably higher specific activity than the homologous tracer (see below).

THE LABELED LIGAND

The labeled ligand is often called "tracer" for short. It is not an entirely correct designation, as it implies that really very small amounts, tracer amounts, are used. This is not the case, however, when labeled ligands of low specific activity are employed.

The limit of detection of an assay is determined to a great extent by the specific activity of the labeled ligand. It is not possible to use ^{14}C in RIAs; the specific activity of ^{14}C-labeled compounds is too low. The most commonly employed isotopes in this field are ^{3}H (β-emitter) and ^{125}I (γ-emitter). The theoretical maximum specific activity of a compound containing two atoms of isotope per molecule is approximately 58 Ci/mmole for ^{3}H

and 4,340 for ^{125}I. It thus seems obvious that ^{125}I should be preferred, if possible. However, the half-life of this isotope is very short (60 days, compared to 12 years for ^{3}H), which means that the labeled ligand must be freshly prepared with short intervals. A few laboratories prefer the use of ^{125}I-labeled prostaglandins today (Ohki et al., 1974; Maclouf et al., 1976). Most scientists, however, prefer to use the more stable ^{3}H-labeled compounds.

Many ^{3}H-labeled prostaglandins are commercially available. The first available labeled compound was [9β-^{3}H]$PGF_{2\alpha}$ with a specific activity of 5 to 15 Ci/mmole. This compound was prepared by reduction of PGE_2 with $NaB^{3}H_4$. The specific activity is far from the theoretical maximum (29 Ci/mmole for one ^{3}H per molecule) because not all the molecules are labeled. The use of this compound in $PGF_{2\alpha}$ assays cannot be recommended.

Later, several other ^{3}H-labeled prostaglandins have appeared on the market. Prostaglandins of the "1" series are conveniently prepared by reduction of the Δ^5 double bond from corresponding "2" prostaglandins with tritium gas. Thus two ^{3}H are introduced per molecule; furthermore, as $^{3}H_2$ is available in pure form, all prostaglandin molecules become labeled. In fact, the resulting specific activities often far exceed the theoretical maximum, and the compounds are sometimes reported to have specific activities of more than 100 Ci/mmole. This indicates that the ^{3}H is found in more than two positions per molecule. Labeled prostaglandins of the "2" series are possible to prepare by an analogous reduction of the Δ^{17} double bond of corresponding "3" prostaglandins. This is often done in our laboratory (Gréen et al., 1967, 1973). The resulting [17,18-$^{3}H_2$]-labeled prostaglandins have been found to have specific activities close to the theoretical maximum (around 50 Ci/mmole) (see Chapter 2). If, for example, [17,18-$^{3}H_2$]PGE_2 is prepared, it is possible to prepare tritium-labeled $PGF_{2\alpha}$ and $PGF_{2\beta}$ with even higher specific activities, *viz.*, by reduction with $NaB^{3}H_4$; the resulting compounds are then [9β,17,18-$^{3}H_3$]$PGF_{2\alpha}$ and [9α,17,18-$^{3}H_3$]$PGF_{2\beta}$.

[17,18-$^{3}H_2$]PGs of the "2" series are not commercially available. However, highly radioactive prostaglandins of this series

have recently appeared that were prepared from [5,6,8,9,11,12,14,15-$^{3}H_8$]arachidonic acid. The specific activities of these products range from 100 to 200 Ci/mmole, and they contain seven (PGE_2, PGA_2, PGB_2) or eight ($PGF_{2\alpha}$) tritium atoms per molecule. These compounds have been prepared biosynthetically from purified prostaglandin synthetase preparations, and so the endogenous contribution of prostaglandins is minimal.

This approach—biosynthetic conversion of $^{3}H_8$-arachidonic acid by purified prostaglandin synthetase—can be recommended to anyone who prefers to synthesize his own labeled ligand. Using this precursor and a suitable source of enzyme, it is of course also possible to prepare TXB_2 and mono-O-methyl TXB_2 with high specific activities (Granström et al. 1976*a,b*).

The labeled prostaglandins commercially available today are: PGE_1, $PGF_{1\alpha}$, PGA_1, PGB_1 (all labeled with tritium in the 5,6 positions); PGE_2, $PGF_{2\alpha}$, PGA_2, PGB_2 (seven or eight tritium atoms as mentioned above); and two metabolites, 15-keto-13,14-dihydro-PGE_2 and 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (labeled in the 5,6,8,11,12,14 positions). The companies that sell the labeled products are New England Nuclear (Boston, Mass., U.S.A., and Dreieichenhain, West Germany), and the Radiochemical Centre (Amersham, England); both also sell [$^{3}H_8$] arachidonic acid.

Most other metabolites are not yet commercially available but must be prepared in the scientist's own laboratory. No particular problems should be encountered if the metabolite can be prepared synthetically from a suitable precursor, for example, MnO_2 oxidation of a prostaglandin to the corresponding 15-keto compound (see p. 56). If they have to be prepared biosynthetically, the same problem arises as in the preparation of standard substances; it is mandatory that the endogenous contribution is suppressed. This is the case with all metabolites that have to be prepared *in vivo* (e.g., 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid) or with crude enzyme preparations *in vivo*. Convenient solutions to this problem were suggested above.

Use of Heterologous Tracer

If the appropriate labeled ligand cannot be purchased or prepared, a possible approach is to employ a different compound as tracer in the RIA, a so-called heterologous tracer. (This is in contrast to what is recommended for raising the antiserum or preparation of standards, where the proper compound must be used.) Heterologous tracers have often been employed in prostaglandin RIA, particularly earlier when few labeled substances were available. Labeled prostaglandins of the "1" series were often used in RIAs for a prostaglandin of the "2" series (Jubiz et al., 1972; Zusman et al., 1972) or 15-keto prostaglandins in RIAs for 15-keto-13,14-dihydro prostaglandins (Levine and Gutierrez-Cernosek, 1973). As was mentioned above, a similar possibility is to use a tetranor-monocarboxylic acid (5α,7α-dihydroxy-11-ketotetranorprostanoic acid) for labeled ligand in an assay for the corresponding dioic acid.

The prerequisite for using a heterologous tracer is that the cross reaction with the compound in question should be high. If this is the case, it may even be advantageous to use the heterologous compound, as the sensitivity of the assay may be increased somewhat. In this particular field, the main advantage of the above-mentioned examples is that they can all be prepared either biosynthetically under circumstances which do not lead to endogenous dilution by unlabeled substance, or even synthetically, which is of course the superior alternative.

If the cross reaction with a heterologous tracer is low, however, the compound is not suitable. An example of that is the use of ^{3}H-PGF$_{2\alpha}$ in assays for 15-methyl-PGF$_{2\alpha}$.

If the desired labeled compound is not commercially available and cannot easily be prepared by introducing tritium atoms into a related prostaglandin, a further possibility is to conjugate the unlabeled substance to compounds such as tyrosine methyl ester, histamine, or tyramine, and then iodinate these compounds with ^{125}I (Ohki et al., 1974; Maclouf et al., 1976; Sors et al., 1977). As mentioned above, they offer the advantage of having

considerably higher specific activities than the common tritium-labeled ligands. The most commonly employed method for the preparation is the chloramine-T method (Hunter and Greenwood, 1962; Maclouf et al., 1976). This approach is possible because the antibodies, as mentioned earlier, do not recognize the site of coupling, *viz.*, the structures close to the carboxyl group.

For the same reason, it is equally possible in most RIAs to use the methyl ester instead of the free acid of the labeled ligand; the cross reaction should be close to 100%. An exception to this is the RIA for the dioic acid metabolite, in which one of the carboxyls is well recognized by the antibody. Methyl esters are sometimes more easily prepared and may be somewhat more stable on storage than the corresponding free acid.

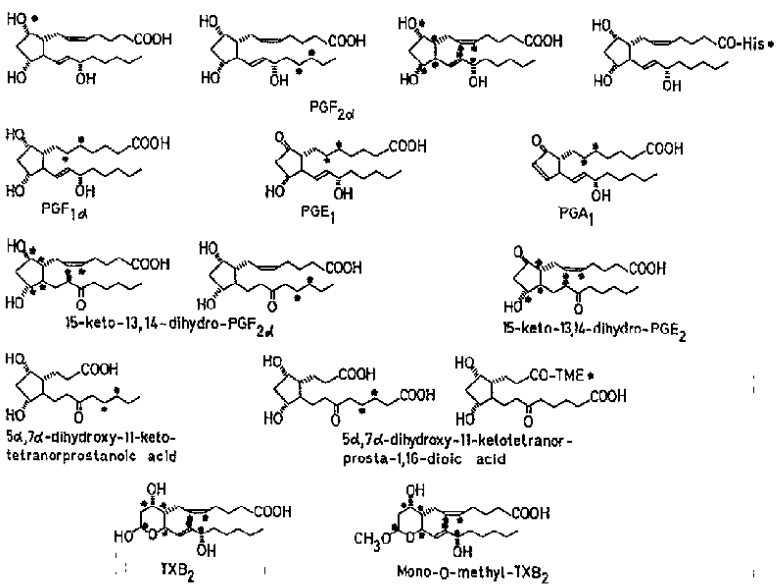

FIG. 9. The position of label in compounds commonly employed as labeled ligands. (His) Histamine. (TME) Tyrosine methyl ester.

Figure 9 shows a summary of commonly employed labeled ligands.

Check of Purity

Any labeled compound used as ligand in the assay must be frequently checked for purity as these substances are not infinitely stable. First, the substance itself may be liable to decomposition (e.g., PGE compounds); and second, the irradiation damage may become considerable. It is often seen that in a RIA that has previously worked satisfactorily, the "maximum binding" after some time starts to decrease. The reason in most cases is degradation of the labeled ligand, which must then be purified or freshly prepared.

Thin layer chromatography in suitable solvent systems is a convenient way of checking the purity; however, purification should not be performed with this kind of chromatography. The reason is that these minute amounts of highly radioactive substances rapidly decompose on a thin layer plate, and the resulting "purified" compound may thus contain an even higher percentage of decomposition products than did the original substance.

The purity check should be performed not once but repeatedly. We recommend a regular check at least once a month for most compounds, and at least once a week for arachidonic acid. These highly radioactive tritium-containing substances are apt to decompose spontaneously; the β-radiation has a strong tendency to disrupt double bonds. To prevent this as much as possible it is generally recommended to store the labeled substances in a solvent such as benzene. In most RIA publications in this field, it is not stated that the commercially obtained labeled substance was subjected to careful control before use. The reader inevitably gets the impression that the ampul was just opened and its contents used as such. This may give strongly misleading results, as the statements from the manufacturer may be highly optimistic.

RAISING THE ANTIBODY

Choice of Animals

The most commonly employed species in this field are the rabbit and the guinea pig. Rabbits are hardy, relatively resistant to various intercurrent infections that might otherwise spoil the results, and easy to cage. They are also easy to bleed, and 30 to 40 ml blood can be collected at frequent intervals without causing the animal any harm. Guinea pigs are smaller and thus require less space and somewhat less immunogen. They are, however, more difficult to bleed than the rabbit, and only relatively small blood volumes can be taken on each occasion.

Other species sometimes employed are the rat, chicken, monkey, and small ruminants like the sheep or the goat. In general the larger species (e.g., the sheep and goat) are better for production of second antibodies, that is, precipitating antisera for use in the double-antibody techniques. These are often required in relatively large volumes. Chickens often give rise to good antisera; however the avian γ-globulins differ somewhat in their physicochemical properties from the mammalian ones, and this may necessitate modifications in the precipitation techniques of the assays.

Special problems may exist in the prostaglandin area. Some species (e.g., the rabbit) have an isomerase in their plasma that catalyzes the conversion of PGA to PGC compounds (Jones et al., 1972). This enzyme has been supposed to be the reason for their failure to produce antisera against PGA compounds (Levine et al., 1971). It has been suggested that the use of animals lacking this isomerase (e.g., sheep or guinea pig) might result in better antisera. However, the production of excellent antisera for PGA_1 by the rabbit was recently reported (Zia et al., 1975).

In conclusion, there is currently little evidence pointing to the clear superiority of one species over any other. It is likely that any species which suits the scientist for practical reasons (space,

economic considerations) can be used. As a rule of thumb, animals that have not responded after 3 months immunization should be disposed of and other animals tried instead. If no antibody production has been obtained during a year of attempts, a new species could be tried.

Before leaving this topic, one more problem should be mentioned. It is often seen that if the animals contract infections during the immunization the titer of the induced antibody decreases considerably. Even if the animal survives, much effort will be wasted. Mortal infections are unfortunately not uncommon. Thus as these extremely valuable animals are intended for use during very long periods, they must be of the highest available quality, *viz.*, specific pathogen-free (SPF)-raised. This excludes the possibility of some of the more common diseases. Furthermore, these animals should be kept isolated, with restricted admission to their room. No other animals should be kept in the same room.

Dosage of Immunogen and Preparation of Emulsion

The antibody response is almost independent of the amount of immunogen given, if only a certain minimum quantity is exceeded. Below this amount, no antibodies are produced or an antiserum is obtained that has a titer too low to be practically useful. Larger amounts of immunogen than necessary do not give rise to better antisera; on the contrary, the avidity of the resulting antibodies may decrease. Of PG–BSA conjugates prepared according to the common methods (see above), not more than 0.5 to 5 mg is recommended for the primary inoculation, and even smaller amounts can be used for subsequent injections.

Injection of immunogen without an adjuvant is generally wasted effort. The adjuvant stimulates the phagocytic activity in the body; this is essential for the production of an immunoresponse. The most commonly employed adjuvant by far is Freund's complete or incomplete adjuvant. The incomplete type consists of an extract of mycobacteria in mineral oil; the complete type contains whole mycobacteria in addition. (If Freund's complete adjuvant is chosen, take care that the mycobacteria are not left in the ampul; they are generally present as a very small sediment at the bottom.)

The emulsion should be the water-in-oil type, the water phase preferably one-third to one-fifth the emulsion volume (v/v). In our own immunizations the protein conjugate (0.5 to 5 mg) is dissolved in 0.5 ml water, and 1 ml Freund's complete adjuvant is added. The emulsion is prepared by repeated aspiration of the mixture into a syringe and ejection through a medium-thin needle into a medical flask. This is repeated at least 20 times or until the emulsion is white, stable, and viscous. A similar and often recommended method to prepare small volumes of emulsions is to join one syringe containing the aqueous phase to another syringe containing the adjuvant by means of a double hub connector and then rapidly pass the contents to and fro many times. Equipment specially designed for the preparation of emulsions also exists, so-called emulsifiers. These are expensive and in our opinion not necessary, at least not for the preparation of small emulsion volumes.

It should be pointed out that the conjugate ought to be dissolved in distilled water rather than saline. Although saline is used routinely as a vehicle for all kinds of injections, in this case it is not suitable. Adding sodium chloride (or any other salt) to water–oil emulsions is a convenient method of breaking such emulsions.

Route of Administration

Many routes of administration have been used, for example, intraglandular (lymph nodes), intraarticular, intradermal, intramuscular, intraperitoneal, subcutaneous, and intravenous. For strong immunogens the site of administration does not seem to be of importance for the final result. It is known that the predominant site of antibody production is determined to some extent by the site of injection, regional lymph nodes becoming

the most active when administration is local, and the spleen and lung being more active when intravenous injection is performed.

The most commonly employed are the subcutaneous and intramuscular routes, and recently also intradermal injection at multiple sites, which requires smaller amounts of immunogen (Vaitukaitis et al., 1971). A convenient way of administering the emulsion to a rabbit is to inject it subcutaneously and intradermally at multiple sites close to the lymph drainage in the flank area. Footpad injections are also commonly used, but we do not employ this method because of the intense pain it causes the animal.

After several days abscesses can be detected at the injection sites. If the injections have been carried out properly, these are sterile abscesses and do not have to be treated unless they seem to embarrass the animal.

Immunization Schedule

Injections should not be given too frequently. Most workers in this field give the animals weekly injections of the immunogen, at least early in the schedule. This regimen may not be necessary as one of the properties of an oily emulsion is to allow a continuous slow release of antigen for a period of several weeks. After an initial inoculation period with two or three injections, it is advantageous to let the animal rest for several months and then give a booster injection. The animal should be bled 7 to 14 days after this last injection. Such a schedule often leads to the production of high-avidity antisera. With more frequent injections and without the rest period, the antibody titer may become higher; however, this increase in titer sometimes occurs at the expense of other valuable properties of the antiserum: the avidity and possibly also the specificity.

It may be advisable to bleed the animal after the initial immunization period, *viz.*, after the second or third injection. It is generally possible to detect an immunological response at this

early stage and thus give booster injections only to the responsive animals, which saves unnecessary use of conjugate.

Antiserum or Antiplasma?

In the RIA literature, the word "antiserum" is the standard designation for the crude antibody preparation obtained from the animal; the reason of course is simply that the collected blood is always allowed to clot and so serum is obtained. For prostaglandins this is sometimes not the optimal method. In many cases it is prefcrable to include an anticoagulant in the tubes for blood collection and thus obtain the "antiplasma" instead. This approach is suitable for animals immunized against compounds that are known to be released during blood clotting. Thus blocking of antibody binding sites by this endogenously produced material is minimized, which is particularly important in raising antithromboxane antibodies but may also be advantageous with antibodies against primary prostaglandins. A further inhibition of this deleterious endogenous production may be obtained by including indomethacin or some related compound in isotonic saline in the blood collection tube (final concentration in the collected blood is 10^{-5} M) or even *in vivo* treatment of the animal with prostaglandin synthetase inhibitors for some time prior to the bleeding.

Bleeding the Animals

Rabbit. Blood collection is easily performed in the rabbit. The vessels of the ear are easily identified (one central artery and two marginal veins, the two veins being suitable for this purpose), and the thin hairs of the covering skin can be removed simply by pulling. Small amounts of blood (approximately 1 ml) for determination of antibody titer can be collected after a small incision across the marginal vein or by puncturing the ves-

sel with a needle. Pressure must be applied at the base of the ear (squeeze gently between thumb and forefinger).

There are several methods for collecting larger amounts of blood. From an animal with a body weight of 3 kg, approximately 30 ml can be collected every 2 to 3 weeks; if only a single collection is intended, as much as 50 ml can be taken without causing the animal any harm:

1. Apply xylene on the skin over the central artery and one marginal vein and then collect blood through a needle. No aspiration is necessary. The needle protects the blood from coming in contact with the xylene, which otherwise causes hemolysis.

2. Use the xylene as above but cover the skin over the vein with a thin layer of vaseline or melted paraffin wax (use paraffin wax with a melting point of 40° to 45°C in order not to scald the animal). Then cut across the vein through the wax with a scalpel or a razor blade. This method gives a fairly good, constant blood flow, and the paraffin also protects the blood from contact with the xylene.

Note: xylene induces edema, and it is thus necessary to remove the xylene as soon as the bleeding is finished. Washing with 70% ethanol is recommended.

Both these methods are easy to perform and allow the collection of an exact amount of blood. This is advantageous in case the blood must be collected directly into tubes containing fixed amounts of, for example, heparin and/or indomethacin to give certain final concentrations of these substances.

3. A commonly used method is to collect blood with a vacuum reservoir flask. This flask can be made from a 500-ml Erlenmeyer flask simply by making three extra outlets: one for attaching a tightly fitting test tube that collects the blood, one connected to a vacuum pump, and the third for manual control of the vacuum, which should be intermittent. A small incision is made across the marginal ear vein, and the whole ear is put inside the flask through the main opening, which is pressed against the skull of the animal. Blood flows freely from the incision when the vacuum increases and is allowed to run into the test tube. The

method is simple and efficient but has two drawbacks. First, rapid addition of anticoagulant or prostaglandin synthetase inhibitors to the blood is difficult because the blood initially spreads over the inner surface of the flask and takes some time before it enters the test tube; second, the tympanic membrane of the animal easily ruptures, which may lead to serious middle ear infections later. The whole ear also becomes severely bruised by this method if the vacuum is not carefully controlled.

4. Cardiac puncture is efficient but requires a good deal of skill, and we do not recommend it when dealing with valuable animals that are to be used for long periods.

For a final death bleeding, cardiac puncture or bleeding through one of the carotids is recommended. For surgical dissection of the carotid, slight ether inhalation plus local anesthesia are required. The vessel is punctured and a catheter inserted.

Guinea pig. This small animal is more difficult to bleed. Small amounts (0.25 to 0.5 ml) of blood can be obtained from the ear vein in the same manner as with the rabbit. Another possibility is to cut one claw close to its base through the pulp; the claw should be gently warmed before cutting.

Larger amounts of blood can be obtained by cardiac puncture or from the jugular or the saphenous vein after some surgical dissection. Cardiac puncture is probably the most common method in this species; it is difficult, however, and may result in a fatal cardiac tamponade if performed by the unexperienced scientist.

No more than 7 to 10 ml blood should be taken on each occasion from an average-sized guinea pig weighing approximately 500 g. Moreover, the animal in such cases should not be bled more often than once a week.

Rat. Rats can be bled quite easily by puncturing the large tail veins.

Small ruminants (sheep, goat). These animals are very easy to bleed by puncturing the jugular veins. It is recommended that the hair from the animal's neck be shaved before puncture. For these comparatively large animals, we always use the Vacutainer

system (Becton-Dickinson). It is possible even to add a solution containing, for example, a prostaglandin synthesis inhibitor to these tubes by perforating the rubber stopper with a needle and syringe, without losing the vacuum.

Assessment of Antibody Titer

A suitable working dilution of the antiserum (or antiplasma) is one that binds 40 to 60% of the labeled ligand in the absence of unlabeled antigen. The use of excess antibody should be avoided as this results in a decrease in sensitivity of the assay: All binding sites must be occupied before the unlabeled material can compete with and displace the labeled antigen. This effect on the sensitivity is demonstrated in Fig. 10, where standard curves were run with an increasing excess of antiserum.

The whole standard curve need not be analyzed for each serum dilution to establish the optimal working titer. A preliminary experiment can give a good indication of this titer: Doubling dilutions (e.g., from 1/100 to 1/51,200) of the antiserum are incubated with a constant amount of labeled antigen, and the amount of radioactivity in the antibody-bound or the free

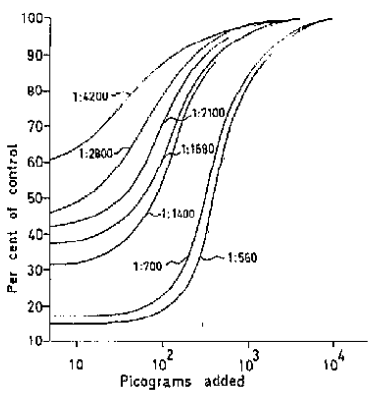

FIG. 10. Loss of sensitivity in a $PGF_{2\beta}$ RIA with the use of an increasing excess of antiserum.

fraction is determined after separation. A closer determination, preferably with the complete set of standards, is subsequently carried out in the range of dilution that binds 40 to 60% of the labeled ligand, and the most suitable working dilution is chosen from these curves.

As was briefly mentioned in the previous section, the working titer of the antibody preparation sometimes seems to change. This is only an apparent change, as the antisera are very stable on storage. The reason is almost always found in one of the other reagents. If the maximum binding decreases, degradation of the labeled ligand is the most likely explanation. If the whole standard curve indicates a lower sensitivity of the assay, the error may reside in the standard dilutions. (With a decreased displacement, the curve shifts to the right caused by degradation of standard. With an apparently increased displacement, the curve shifts to the left caused by contamination of standard solutions, for example, plastic material from the pipette tips.)

However, sometimes no obvious explanation can be found for a change in the working titer of the antibody preparation. This change then appears to be "real," and the working dilution must simply be adjusted to give the normal maximal binding of 40 to 60%. We have often seen an apparent rise in titer of several antisera. For instance, the working dilution of our anti-15-keto-13,14-dihydro-$PGF_{2\alpha}$ antiserum was 1:700 early in 1972. Two years later the titer (from the same bleeding) was 1:3,000, and now (1977) it is 1:24,000. The tracer preparation has been of approximately equal quality during this time, and we believe the explanation for this phenomenon is deterioration of endogenous 15-keto-13,14-dihydro-$PGF_{2\alpha}$ in the antiserum, thus yielding gradually more binding sites on the antibodies available. This phenomenon stresses the advantage of treating the animals with prostaglandin synthetase inhibitors prior to bleeding.

EXTRACTION AND PURIFICATION OF SAMPLES

During the earliest years of prostaglandin RIAs, most scientists extracted their samples and often also purified them ex-

tensively (Caldwell et al., 1971; Jaffe et al., 1971). Later it was questioned whether this is really necessary, and the advantages and drawbacks of using unextracted versus extracted samples has been discussed (Jubiz et al., 1972; Patrono, 1973; Youssefnejadian et al., 1974).

The reasons for extraction are:

1. Concentration of the substance (the level of the substance may be below the limit of detection in the assay).
2. Dissociation of the prostaglandin from albumin.
3. Removal of undesirable factors that interfere with the assay.
4. If a further purification is carried out: group separation of prostaglandins, which may be necessary if the antiserum is nonspecific and cross reacts extensively with other prostaglandins that occur in the sample.

The drawbacks of extraction of samples are:

1. The capacity of the method is greatly diminished.
2. Problems with estimation of recovery appear.
3. The recovery may be low and variations are inevitable.
4. Introduction of unspecific interfering factors by the procedure (solvent impurities, leakage from columns, impurities in gas used for evaporation).
5. Practical problems: some samples form emulsions upon shaking with organic solvents, and so forth.

Thus is it necessary to extract samples? We do not feel that it is in many cases. On the contrary, extraction may introduce more problems than it solves (see below).

The albumin binding of prostaglandin in blood is no major problem. This binding is weak and freely reversible; and in competition with an antibody of high avidity (K value is generally around 10^9 to 10^{10} M^{-1}), almost no prostaglandin would remain albumin-bound (Unger, 1972). In fact, we often carry out the whole assay in a solution of albumin and have not seen any interference by this protein in the assay.

If the levels of the compound are too low to be measured directly, it is often possible to increase the sensitivity of the assay by other means: using a labeled ligand of higher specific activity, a heterologous tracer, delayed addition of tracer, a higher dilution of the antiserum. In general, small variations in the assay conditions may give the desired increase in sensitivity without introducing an extraction (and purification) step.

The recovery problem is difficult to solve. The addition of a small amount of the labeled compound is necessary for estimation of recovery. This amount has to be small not to interfere with the later assay, and thus the determination of recovery cannot be made with a high degree of accuracy. Some scientists prefer to circumvent this problem by using a different labeled prostaglandin as a marker, a compound with similar extraction properties but not cross-reacting in the assay. However, the extra radioactivity may still interfere (this labeled compound is present in the free fraction mostly). Furthermore, this approach may be hazardous since the properties of the prostaglandins may differ more than expected. This is particularly the case if a chromatographic step is included, in which case the added labeled marker and the desired prostaglandin may actually separate to some extent.

The measurement of recovery involves more pitfalls. A commonly employed procedure that introduces a large error into the calculations is the following: The extract, or chromatographic effluent containing the substance, is evaporated in a scintillation vial. A small amount of RIA buffer is added (e.g., 0.5 ml), and 0.1 ml of the "solution" in duplicate is assayed in the RIA. The remainder in the vial is subjected to addition of several milliliters of scintillation fluid, and the vial is counted. The result of such an approach is very misleading, for the following reason.

The evaporated extract in many cases is not completely soluble in a small volume of an aqueous medium. This is particularly the case with crude extracts. Thus only a small proportion of the sample is found in the buffer and gives erroneously low

values in the assay. The undissolved major part of the sample is allowed to remain in the vial, and a large volume of a nonpolar solvent mixture, the scintillation fluid, is added, which immediately and completely dissolves the rest of the sample. The result is a higher-than-actual recovery figure and a lower-than-actual value in the assay. Both these deviations from the actual situation tend to give confidence to the method, particularly if the assay deals with plasma prostaglandins, which in most cases tend to give too high values in RIAs.

To avoid this common and large error, the portion of the sample intended to be counted for recovery estimation must not be allowed to remain in the vial but must be transferred in the buffer solution to another vial before adding the scintillation fluid.

An increasing interest has recently been taken in measuring prostaglandins in various tissues. These measurements are most uncertain: It is difficult to obtain the tissue sample in a way that prevents artifactual formation and/or metabolism of the compounds—the sample must be deep-frozen within a few seconds after removal—and it is even more difficult to obtain any idea of the recovery of prostaglandins after an extraction step. Some add the labeled substance also to these samples and report recoveries after the extraction. This is not a reliable method, however. To be reliable, one must ascertain complete equilibration between the added label and the endogenous prostaglandin, and this is not likely to take place or, at least, is difficult to ascertain. Furthermore, time elapses before the extraction starts, and so a good deal of artifactual formation of prostaglandins and even metabolism of them is likely to take place, and the finally measured levels do not represent the true values.

Performance of the Extraction

Many methods have been employed for extracting prostaglandins and their metabolites from biological material. None of these methods seems to be definitely superior to the others; the

reported recoveries of prostaglandins are comparable in most of the methods. Some scientists prefer to start the extraction with a nonpolar solvent (e.g., hexane) at a neutral pH to remove neutral lipids. Others omit this step and acidify their samples directly. Acidification is commonly carried out with dilute HCl or an organic acid such as citric acid. The extraction should generally be carried out at a pH around 4 to ensure complete transfer of the prostaglandin to the organic solvent. However, when extracting PGE compounds, a somewhat higher pH may be recommended to prevent the conversion into PGA compounds. When choosing the solvent or solvent mixture, the scientist must consider the polarity of the desired compound. For polar prostaglandins such as the F compounds, a nonpolar solvent (e.g., ether or chloroform) is not suitable as the recovery would be too low after this stage. These solvents may suit nonpolar prostaglandins such as the compounds of the A and B type better. For PGFs, ethyl acetate may be preferable. The literature offers the choice between many procedures (see also Chapter 2).

The volume of the sample may be of importance for the results. Dray et al. (1975) pointed out that values obtained from samples smaller than 3 ml may not be reliable and recommended a volume of 5 to 10 ml for routine use. In spite of this, most reports describing extraction procedures deal with samples of 0.5 to 2 ml. However, the use of large sample volumes also has some drawbacks: It necessitates the use of larger solvent volumes with the risk of introducing impurities; it precludes the use of disposable glassware; and finally, it reduces the practicability of the RIA considerably.

An extraction may be conveniently performed (e.g., in disposable glass tubes) where the sample is vortexed together with the solvent. The phases are separated either by removal of one of them with a capillary pipette or by freezing the water phase and decanting the organic, upper phase into another tube for evaporation (the latter method cannot be used in chloroform extractions, where the organic phase is the lower one).

Emulsions often occur during extraction of plasma samples,

and this phenomenon constitutes a great problem. Many of them cannot be overcome simply by centrifugation or freezing the water layer. Furthermore, it is impossible to predict which samples will give emulsions and which will be extracted without problems. Even in a series of plasma samples obtained from the same subject during a short time, all samples may not behave identically in this respect. A large volume of the organic phase is trapped in the foamy interphase, which reduces the recovery considerably. Emulsions are completely avoided if the extraction is performed using the neutral resin Amberlite XAD-2 instead. This method is superior to other extraction methods in most respects but unfortunately cannot be used in connection with RIA owing to the introduction of a strongly interfering substance from the resin (see below).

After extraction, the organic phase must be washed neutral. This important step is omitted in most published procedures, and thus there is the risk that the extracted samples are assayed at a lower pH than the standards (see below).

The subsequent evaporation of the organic solvent can be performed in different ways, depending on the volatility of the solvent. The most commonly employed method is evaporation under a stream of nitrogen, argon, or air at room temperature or a slightly elevated temperature. We found that this step also introduced some impurities into the samples, even with extensively washed gas, and so we omitted it. Spontaneous evaporation to dryness at room temperature without any gas stream was the preferred method in our laboratory. Elevated temperature should be avoided because of the risk of decomposition of these minute amounts of prostaglandins when the sample has reached dryness. The evaporation can also be performed at reduced pressure with the aid of either a water evaporator or a vacuum pump.

Many scientists interrupt the purification procedure at this stage and assay the extracts; others perform subsequent chromatography (generally silicic acid or thin layer chromatography). If the chromatography is omitted, the sample is now "dissolved"

in the RIA buffer. Unfortunately, the solubility of the prostaglandin in the buffer is not quite the same in a lipidic extract as it would be for the pure compound. Thus a considerable part of the prostaglandin fraction may be lost at this stage if care is not taken that the added buffer volume really dissolves it (see above). The problem hardly arises if the neutral lipids have been removed by an initial extraction at neutral pH; but it may be considerable if this has not been done, and the extract was obtained, for instance, from a lipid-containing postprandial plasma sample.

The most commonly employed chromatographic procedure in this field is silicic acid chromatography, preferably in microcolumns, for group separation of PGA/B, PGE, and PGF compounds. The solvents used for this separation are mixtures of ethyl acetate, benzene, and methanol in varying proportions. The method by Caldwell et al. (1971) has been most widely employed, either as such or after minor modifications (Auletta et al., 1974; Dray et al., 1975). Whichever method the scientist prefers, it is necessary to evaluate its separation properties first by using larger amounts of the labeled prostaglandins under study to find the optimal conditions: column size, volumes of effluent of each solvent mixture, rate of flow, and so forth. It must be pointed out that the chromatogram published by Caldwell et al. (1972), Auletta et al. (1974), and others, shows the elution pattern under ideal conditions. The method often does not give this 100% separation of the three groups of prostaglandins as indicated in the picture. The chromatogram published by Van Orden and Farley (1973) gives a somewhat more realistic description of the events.

After a chromatographic purification of the prostaglandins, the samples are again taken to dryness. The now fairly pure samples are considerably more easily dissolved in buffer, and this step does not constitute a problem as with the lipid-containing extracts. Nevertheless, the solubility must be checked here also.

The recovery after various extraction and chromatographic procedures generally ranges from 60 to 80%, depending on

which prostaglandin was extracted. Extreme recovery values may be as low as 40% or as high as 90%. It is of course desirable that the method gives a high recovery, preferably at least 70%, but it is even more important that the figure is reproducible. Some scientists prefer to add tracer to all their samples to obtain an exact measurement of the recovery after each extraction; others practice the method initially with tracer included until the variation is minimal, and then exclude the tracer from samples intended to be measured in the RIA, calculating with an average figure for the recovery. Both methods have their drawbacks. Even a very small amount of radioactivity, which cannot be exactly the same at the final stage of all samples, will disturb the later performed RIA; on the other hand, if the tracer is excluded from the extractions, the samples that obviously deviate from the average (accidental losses, emulsion formations) cannot be adequately measured.

Parallelism Test

One very important experiment must always be carried out both with extracts and unextracted samples, *viz.*, a test for *parallelism* in the RIA. Every sample, whether extracted or not, must on dilution yield a curve parallel to that of the standard. Lack of parallelism indicates a nonspecific effect. However, even if perfect parallelism is obtained, this does not completely rule out the presence of nonspecific, interfering factors. In the early phase of a study, where the behavior of the sample in the assay is evaluated, this dilution test must be performed with at least 8 to 10 sample dilutions. Later in the study when parallelism has been established, this is not necessary (provided no changes have been introduced into the procedure), but a minimum requirement is still to assay each sample at two dilutions; we generally prefer four. If the four final values from these measurements do not agree with each other, the factor responsible for the disagreement must be sought and eliminated.

The few methods that deal with the measurement of urinary

metabolites of prostaglandins have all been developed for assaying unextracted urine (Ohki et al., 1974, 1975, 1976; Cornette et al., 1975; Granström and Kindahl, 1976*b*). Due to the comparatively large amounts of the dioic acid present in this fluid (in the order of magnitude of 10 μg/24 hr) (Hamberg, 1973), the urine can be diluted considerably prior to assay. Thus although it is likely that urine contains a large amount of substances that may interfere with the assay, it is possible to find a wide range of dilutions where perfect parallelism is obtained. It is likely that the interference by the other urinary constituents does not come into play until considerably higher concentrations are reached.

If dilution in this case had proved not to be sufficient, great problems would have arisen; because of the high polarity of the tetranor dioic acid, even ethyl acetate is not a particularly efficient solvent for the extraction of this compound. The best alternative is butanol, but this solvent has a high boiling point and is thus difficult to evaporate. Amberlite XAD-2 is of course superior under other circumstances but cannot be used here, as mentioned.

The Blank Problem

BIOLOGICAL BLANK

Because of the many interfering substances that may occur in the biological material and may even be concentrated during the extraction procedure together with the desired prostaglandin, it would be advantageous to analyze also samples obtained from "blank" material, for example, plasma known to contain no prostaglandins at all. However, in this field no such thing as a true biological blank exists as yet. Various approaches have been made in the past, for example, the use of plasma from hysterectomized subjects or from subjects treated with prostaglandin synthetase inhibitors. The uterus, however, is not the only organ that produces prostaglandins—it is not even the

major one. Subjects also react differently upon treatment with prostaglandin synthetase inhibitors (Hamberg, 1972); furthermore, their plasma then contains a variable and unknown amount of the drug in question, which is also likely to be concentrated during an extraction procedure and may disturb the assay.

More recently, artificially produced blanks have been used. The biological fluid has been either extensively dialyzed or "stripped" by treatment with charcoal. These "blanks" must be viewed with some suspicion. If an extraction procedure yields nonspecific material that gives parallel inhibition, then this material too may be removed by the initial dialysis or adsorption procedure together with the prostaglandins. Thus the difference in "prostaglandin level" obtained by analysis of the biological material prior to and after the adsorption does not necessarily reflect only the prostaglandin content.

PROCEDURE BLANK

Of the multitude of recent reports where RIA is used for prostaglandin measurements, the majority includes some processing of the sample in the procedure, for example, an extraction step. Unfortunately, very few of these papers report the "procedure blank," that is, the result obtained in the assay from a water or buffer sample subjected to exactly the same treatment as the biological samples. This is unfortunate, as even the most rigorously performed extractions and purifications are likely to introduce some interfering material (see examples given in a later section), and the omission of this information makes it difficult for the reader to evaluate the significance of the variations, particularly in the lower levels reported. In the few reports that mention the contribution from the procedure itself (often in the order of magnitude of 5 to 15 pg "prostaglandin" when compared to the standard curve), nothing is mentioned about how this procedure blank was treated in the final calculation of data, thus leaving the reader with the suspicion that this blank value was simply subtracted from the measurements of the unknowns. Sometimes,

this is even frankly stated. This procedure is not correct, however, and introduces a serious error. It is important to realize that the observation of a significant "blank" value inevitably implies a nonspecific effect, either on the binding of antigen to antibody or on the separation system. The magnitude of this nonspecific effect normally depends on the relative concentrations of antigen and antibody. Thus it is not legitimate to assume that blank values may be subtracted from measurements of unknowns, since the magnitude of the correction varies at different points of the standard curve. Only if the interfering factor is completely identical to the prostaglandin in its reaction energy with the antibody will the entire response curve not be distorted but merely displaced.

PERFORMANCE OF THE RADIOIMMUNOASSAY

The risk of contaminating laboratory utensils and solutions with prostaglandins must be constantly kept in mind. Other work with larger amounts of prostaglandins should not be allowed in the same room. We do not store or work with amounts of prostaglandins exceeding 1 μg in the RIA laboratory. For the same reason, disposable glassware should be used as much as possible. Glassware that must be used repeatedly should be washed in the RIA laboratory and not be allowed to come in contact with glassware from other departments.

We prefer to use *glass*ware, even for the disposable utensils. Plastic equipment is less expensive, but we avoid it as much as possible because of the risk of dissolving constituents from this material (see below). The only equipment that must be made of plastic is the disposable pipette tips, since no acceptable alternative exists.

Utensils, Buffers, and Major Equipment

As the final volume in the common RIAs is approximately 0.5 to 1.5 ml, a recommended size for the glass tubes in the

assay is 10 × 75 to 80 mm. Suitable tubes of this size are the "culture" tubes.

It is convenient to use test tube racks that allow a certain distance between the tubes and also keep the tubes in a fixed, vertical position. It is then easy to see if a pipetting error has been made somewhere and one tube contains too little or too much.

It is essential that automatic pipettes with disposable tips are employed for all pipetting. Only the pipetting of duplicates is allowed with the same tip, or the serial pipetting from one solution to a large number of tubes. Several manufacturers provide good automatic pipettes for this purpose, for example, Eppendorff (Gerätebau Netheler & Henz, Hamburg, West Germany), MLA (Medical Laboratory Automation Inc., Mount Vernon, N.Y.), or Biopette (Schwarz-Mann, Orangeburg, N.Y.). For the serial addition of a large volume of a reagent to a large number of tubes (e.g., when polyethylene glycol is used for the precipitation step), a Cornwall syringe with a refill unit is recommended. There are, however, a number of more advanced automatic dispensers for this type of addition, or even for the simultaneous addition of two reagents to the RIA tubes, for example, the Ultro-lab System Diluter (LKB-Produkter, Stockholm, Sweden). Highly advanced automatic pipetting stations also exist, for example, LKB Ultro RIA system. Using these, it is possible to perform several additions with variable volumes simultaneously.

For the preparation of buffers, redistilled H_2O is always used in our laboratory. The presence of a small amount of EDTA may be advantageous, particularly if the double-antibody technique is employed (10^{-3} M is suitable). In most RIAs the pH is in the range of 7.5 to 8.5, as extremes of pH influence the antigen–antibody binding. The ionic strength is important (see below), and the buffer should generally not be more concentrated than 0.01 to 0.05 M. It is often recommendable to include a certain amount of protein in the buffer. The protein plays several important roles; it acts as a carrier in a subsequent precipitation of the antigen–antibody complex, and it coats polar

groups in the glassware and thus prevents the adsorption of immunoglobulins to the vessel walls. The coating effect is always desirable and may be achieved by the addition of any protein, for example, albumin or gelatin. Some precipitation methods require the presence of a certain amount of γ-globulin or dilute serum (the second antibody technique, polyethylene glycol, and several more). By adding dilute serum or even albumin, however, the scientist must be aware of the possible presence of prostaglandins in these reagents. As prostaglandins are to some extent bound by albumin, the presence of this protein in the assay may also necessitate somewhat longer incubation times to ensure sufficient binding of the prostaglandin by the antibody.

The contents of the tubes must be thoroughly mixed after each addition, and for this purpose a vortex type mixer is suitable, such as Whirlimixer (Fisons Scientific Apparatus, Leicestershire, England). Most techniques for separating the bound and free fractions involve centrifugation. For most of these, a refrigerated centrifuge with a high sample capacity, generally around 100 to 200 tubes, is recommended (e.g., Heraeus Christ GmbH, Osterode/Harz, West Germany; Beckman Instruments Inc., Fullerton, Calif.).

Most working solutions must be kept at 4°C as they are used over long time periods. Samples, stock solutions, and undiluted antisera are preferably kept frozen at −20°C, at which temperature they seem to be stable for many years. However, it must be kept in mind that some evaporation may occur even at this low temperature, and the vessels must always be kept well sealed.

Pipetting the Radioimmunoassay

A common setup of the RIA tubes is the following:

Tubes 1 through 3: "Total radioactivity" tubes, or "zero binding" tubes (no antibody present).

Tubes 4 through 6: "Maximal binding" tubes or "blank" tubes (no unlabeled substance added).

Tubes 7 through 24: Standard tubes in a geometrical series

and in duplicates, e.g., 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 pg/tube.

Tubes 25 through *n:* Sample tubes at least in duplicates and in two different dilutions.

It is convenient to add buffer and the substance (standards and unknowns) to the tubes first and carefully mix the contents before proceeding. The protein solution (γ-globulin, albumin, or gelatin) may also be added at this stage if it is not included in the buffer.

The standards are conveniently prepared as a serial geometrical dilution in buffer, starting from, for example, a concentration of 8 ng/ml (= 800 pg/0.1 ml, a commonly used volume for the addition of a reagent) and diluting to 400, 200, 100, 50, 25, 12.5, and 6.25 pg/0.1 ml. These solutions could, for the sake of convenience, be kept in glass scintillation vials with tightly fitting screw caps. The reason for the inclusion of two extra blank tubes in the standard curve (tubes 7 and 8 in the RIA outlined above) is the following. We have often seen that repeated pipetting with plastic pipette tips after some time seems to introduce detectable amounts of some interfering substance into these standard solutions, probably owing to the dissolution of a constituent from the plastic material. This impurity inhibits the antigen–antibody binding in the standard tubes to some degree, thus causing displacement of the whole standard curve upward, which results in falsely low values of the unknowns. (A more detailed discussion on these nonspecific interfering substances appears below.) This phenomenon is quite difficult to detect in its early stages when the undesired displacement is minimal and the final RIA results from the unknowns have not yet become obviously too low. One way of recognizing this contamination early is to include in the standard curve two "samples" of an aliquot of buffer that, in the same way as the standards, has been kept in a scintillation vial and has been subjected to repeated exposure to a number of plastic pipette tips. No difference should be allowed between the final measurements from these two zero tubes (tubes 7 and 8) and those

from the other three maximal binding tubes (tubes 4 through 6 in the example given above) in which fresh buffer is used every time. If a difference is detected, the whole set of standards should be discarded and a new series of dilutions prepared. We generally do not advise the use of the same standard dilutions more than 20 to 30 times.

The next reagent to be added is preferably the antibody, and the tubes are again vortexed and left for some time prior to the final addition of labeled ligand. This is the so-called "delayed addition of tracer" technique, which may increase the sensitivity of the assay somewhat. The amount of the radiolabeled ligand that should be added depends first on the specific activity of this compound. The sensitivity of the assay is decreased if too much substance is added in this step; however, a certain minimum amount of radioactivity is obviously necessary to ensure reliable radioactivity measurements after the assay. These measurements can be made more accurate by increasing the counting time for each sample. However, practical considerations set a limit to this increase, at least if large numbers of samples are to be assayed. We generally do not add more than 4,000 dpm per tube; in practice, this means a counting time of 10 to 20 min per vial in most assays (average counting efficiency is 30 to 35%; binding is 0 to 50%; preset gross counts (see below) are 10,000 counts per vial).

After a final careful mixing of the tube contents, the whole set of tubes is left for a certain time before separation of the bound and free fractions. The incubation time necessary for this step and the previous one ("delayed addition of tracer") must be carefully investigated for each developed assay. Generally the assays are carried out to near-equilibrium conditions, as it would take too long to reach complete equilibrium (probably several days). This time factor does not seem to be as important for prostaglandin RIAs as for the protein hormone assays, but it still must be kept in mind when looking for optimal conditions. This is particularly the case during the final incubation with all the reagents present, prior to the separation of the anti-

body-bound and free fractions. In most assays we prefer to leave the RIA tubes overnight at 4°C before adding the precipitating reagent. In the double-antibody technique assays, the binding of the second antibody to the first antibody–antigen complex is an even more time-consuming procedure, and an incubation time of 3 to 4 days may be required before complete separation is achieved.

The incubation times necessary for each step depend to a high degree also on the temperature. Equilibrium is much more rapid at higher temperatures, and of course incubation could well be carried out at room temperature or even 37°C. However, the energy of the antigen–antibody reaction is lower at the higher temperatures, and for this reason the more sensitive assays are generally carried out at low temperatures and with long incubation times.

Separation of Antibody-Bound and Free Fractions

Separation of the antibody-bound and free fractions is the step where most RIAs differ; it is also the most critical step, where many sources of error appear. A vast number of methods exist. Most of these aim at precipitating the antigen–antibody complex [e.g., the double-antibody technique (immunoprecipitation, addition of an antibody directed against the γ-globulin fraction of the species employed for the production of the first antibody) or a simple physical precipitation of this complex by adding polyethylene glycol, ammonium sulfate, or organic solvents]. A similar method is the use of nitrocellulose membranes, which adsorb γ-globulins. A different approach is removal of the free fraction by adding dextran-coated charcoal. This is probably the most commonly employed method in this field.

The solid-phase type of RIA implies an alternate solution to the problem. Many different types exist. Either the first antibody or an antibody directed against the first one may be solidified by prior coupling to some support (e.g., Sephadex or cellulose beads) or to the inside surface of plastic tubes. The solid-phase

systems have not yet gained widespread use in the prostaglandin analysis field, but a few assays have been developed (Dighe et al., 1975; Fitzpatrick and Wynalda, 1976). This type of assay is often reported to have a somewhat lower sensitivity and reproducibility; however, for future widespread measurements in clinical use it may be suitable, as the required time and cost are considerably less than for other assays.

All the above-mentioned separation methods have serious drawbacks and are often influenced by nonspecific factors that lead to spurious "prostaglandin levels" in the final interpretation of data. The efficiency of immunoprecipitation seems to be utterly variable, depending on protein concentration, presence of certain factors in plasma, presence or absence of EDTA, or some low-molecular-weight interfering substances, and as mentioned above also the time allowed for this step. Excellent summaries of these problems have been published (Morgan et al., 1964*a,b;* Kirkham and Hunter, 1971). The polyethylene glycol (PEG) technique, like the other physical methods, requires a minimum amount of γ-globulin in the tubes to be complete, and the temperature should not exceed 4°C, when PEG is used (Desbuquois and Aurbach, 1971; Van Orden and Farley, 1973). Organic solvents cannot be used in prostaglandin RIAs, as they efficiently dissolve the prostaglandins and "strip" even the bound molecules completely from the binding sites. The stripping effect is a well-known problem also with the dextran-coated charcoal technique. This technique is based on the removal by the charcoal of the free molecules from the incubation; however, if the time allowed for this adsorption becomes too long, the antigen–antibody complex dissociates, more free antigen is adsorbed, and the final radioactivity measurement of the bound fraction becomes too low. This becomes a greater problem as the number of tubes in the assay increases since they obviously cannot all be handled simultaneously.

We have tested several of the above-mentioned techniques and found the PEG method to be the most convenient one. After the final incubation, we add PEG to a final concentration of

12.5%. The tubes, containing 0.7 ml incubation mixture, are cooled on ice for 15 min, and 0.7 ml of an ice-cold 25% solution of PEG in water is added with a Cornwall syringe. The tubes are vigorously vortexed and then centrifuged for 1 hr at 1,400*g*. To ensure complete separation of the antibody-bound fraction, the assay tubes must contain a certain amount of carrier γ-globulin, in our case 1 mg per tube (Desbuquois and Aurbach, 1971; Van Orden and Farley, 1973).

Regardless of the method used, after the antibody-bound and free fractions have been separated, the scientist must decide which fraction to assay for radioactivity. When the dextran-coated charcoal method has been employed, it is most convenient to assay the bound fraction, which is in the supernatant. The supernatant can either be decanted directly into the scintillation vials or, if the volume is too large, a fixed amount can be removed from each tube (use disposable pipette tips for this step). When a precipitation method has been employed (immuno- or physical precipitation), either the supernatant (free fraction) or the pellet (antibody-bound fraction) may be assayed. If the precipitate is preferred, it must not be dissolved directly and assayed as it contains too much supernatant trapped in the pellet. It is recommended that the tubes be allowed to drain upside down on a filter paper or other soft absorbing tissue; the pellets may subsequently be dissolved in suitable buffer, and the bound fraction finally reprecipitated using the same method. After centrifugation the second supernatant is discarded and the resulting pellets are again dissolved in a suitable medium, transferred to vials, and counted. In the solid-phase systems, the supernatant is also conveniently assayed. However, if iodinated tracer is employed, it is also possible to count the bound fraction, which may even be the whole tubes, after the supernatant has been discarded.

As was briefly mentioned above, the separation procedure is the most critical step in the assay and a step where many sources of error appear. Any laboratory setting up RIAs must be capable of using several separation techniques differing from each other

in the physicochemical basis on which they operate. Comparisons must be made between the results obtained with, for instance, dextran-coated charcoal, second antibody, ammonium sulfate, or PEG. The presence of some unfortunately very common nonspecific effects may then become obvious (see below), and the final results will be interpreted with considerably more criticism.

COUNTING THE RADIOACTIVITY

In case a β-emitter, like tritium, is employed, a liquid scintillation system (liquid scintillation counter and a suitable cocktail) must be used. Most liquid scintillation counters today are of high quality. Details of the electronic systems differ among instruments, but they all seem to work with a counting efficiency of at least 60% for unquenched tritium samples, which is generally sufficient. When buying a new instrument for RIA purposes, the following points should be considered.

1. The sample capacity must be high (300 to 450 vials). Overcapacity on the part of the counter rarely occurs when working with RIA.
2. It may be necessary to count the samples at an exact temperature, and therefore the instrument must be equipped with a temperature control.
3. To yield an equal statistical deviation of the counting over the range of the standard curve, the instrument should be equipped with a "preset count," i.e., all vials are counted until a certain number of gross counts has been reached [e.g., 10,000 gross counts (1% deviation)] independent of the time required. This must be combined with "preset time" or "reject level" possibilities, if by accident a vial should contain no radioactivity.
4. The instrument must be equipped with automatic quench correction facilities. When counting RIA vials, internal quench correction should be avoided because it is time-consuming (after counting, all vials must be subjected to the addition of exactly the same amount of a suitable labeled compound followed by

recounting of all the samples) and requires large amounts of labeled compounds. The internal quench correction technique is superior to the external one, however, when working with highly quenched or heterogenous samples.

With large series of vials, it is thus preferable to use a sample channels ratio (i.e., the vial is simultaneously counted in two channels) or automatic external standard channels ratio (the instrument must then be equipped with a gamma source close to the vial). Both these methods are easy to use, but it is necessary to have a calibration curve showing the relationship between the obtained ratio values and the counting efficiency.

In a limited range of quench variation—quenching must always be minimized by choosing the best scintillation cocktail—the relation between ratio and efficiency can be linearized and the dpm value calculated according to the mathematical formula

$$\text{dmp} = \frac{\text{cpm} \times k}{\text{ratio} - a}$$

k = slope of the calibration curve

a = intercept with ordinate

Some new instruments are equipped with automatic dpm counting and even with minicomputers for the final calculation of the content of substance in the unknowns from the standard curve. Instead of using an instrument with a computer, an outlet device may be employed, e.g., magnetic recording or a teletype-writer with a paper-tape perforator for later off-line calculation on a computer. The advantage of using this off-line system is that a bigger computer can be employed, serving many radio-activity counters and providing possibilities for the use of more reliable calculating systems.

Scintillation Cocktails

Many ready-for-use cocktails exist which have a high capacity for water and yet a high counting efficiency, e.g., Instagel (Packard), Bray's solution, Aquasol, Biofluor, Riafluor (New England Nuclear), and Ready-Solv (Beckman). Their capacity for water generally ranges from 10 to 25%. Some form permanent gels if the amount of water exceeds a particular minimal volume. These cocktails cannot be used with certain amounts of water owing to the formation of a two-phase system, but the problem is solved simply by increasing the amount of either the cocktail or the water. When PEG is used for separating the free and the bound antigen, special care must be taken when selecting the scintillation cocktail. In this situation we use either Ready-Solv VI (Beckman) or Instagel (Packard). With Ready-Solv VI, we simply decant the supernatant obtained after centrifuging the RIA tubes (approximately 1.4 ml) into a vial containing 6 ml of this cocktail. After capping and shaking the vial, a clear solution is rapidly formed. When Instagel is used, we remove 1 ml of the supernatant from the RIA tube to a scintillation vial, add 1 ml of water (both operations are easily performed using an automatic diluter, e.g., the LKB Ultrolab System Diluter 2075), and finally add 10 ml Instagel. An emulsion is formed which becomes clear after keeping the vials at 10°C for 24 hr. Instagel is superior, not to say necessary, when working with RIA of unextracted plasma samples. Bray's solution is a third alternative for RIA samples containing PEG, but this cocktail has a slightly lower counting efficiency and is more expensive.

In RIAs where addition of second antibody or dextran-coated charcoal is used for the separation of free and bound antigen, no such problems arise, and the scientist must consider only the water capacity of the cocktail. This is also the case when the bound fraction is preferred for radioactivity measurements in RIAs where PEG has been added, because the amount of PEG present in the precipitate is negligible.

Several manufacturers sell plastic disposable vials, which are very convenient for RIA purposes. For accurate counting and quench estimation, it is necessary to obtain vials with an even wall thickness. Minivials are also available. Using these, the cost for counting is considerably reduced: the price of the vials

themselves is approximately 30% lower than that of ordinary vials, and they do not need more than one-third to one-half the normal cocktail volume. However, the water capacity is of course proportionally reduced, which means that for comparable precision the scientist must increase either the radioactivity or the counting time of each sample. These considerations must be kept in mind when calculating the cost and outlining the practical design of an assay.

Finally, dispensing large volumes of these sometimes highly toxic solutions requires the use of some automatic system that reduces the contact with these chemicals. Several such automatic dispensers exist.

It is always recommendable to consult the manufacturer about each RIA problem for optimal conditions regarding scintillation cocktail volumes, quench correction, calibration curves, etc. Dyer's textbook (1974) on *scintillation counting* is recommended.

MATHEMATICAL HANDLING OF DATA

After the radioactivities of the standards and the unknown samples have been counted, a standard curve can be constructed and the prostaglandin content in the unknowns calculated. As mentioned earlier, it is important to calculate radioactivity in dpm (this is mandatory when dealing with RIA of unextracted samples). In prostaglandin RIAs calculation of the unbound (free) fraction is probably the most common procedure. The following variables are used:

T = total radioactivity per tube in the assay (no addition of antibody; radioactivity in "zero binding" tubes)

B_o = maximal binding in dpm (antibody present, but no addition of unlabeled prostaglandin)

B = dpm of the various standard samples

U = dpm of the unknown samples

A suitable dilution of the antiserum/antiplasma gives $B_o/T \times 100$ = approximately 50%. The corresponding value for the standards ($B/T \times 100$) then gradually increases from this value until finally, with an increasing amount of prostaglandin, total displacement of radioactivity has occurred ($B = T$) (cf. Fig. 6).

In the simplest plot of the standard curve, $B_o/T \times 100$ and $B/T \times 100$ are plotted versus log dose of the concentration of unlabeled prostaglandin (log X). The resulting curve is sigmoid shaped (Figs. 6, 8, and 10) and can be drawn conveniently with a rubber curve fitter on semilogarithmic paper. With this curve, the concentrations of the unknowns can be read from their corresponding $U/T \times 100$ values.

Instead of plotting $B_o/T \times 100$ and $B/T \times 100$, $(B - B_o)/(T - B_o) \times 100$ may be plotted versus log dose. This gives a sigmoidal curve with percent "radioactivity" from 0% (maximum binding) to 100% (total displacement = total radioactivity). Fifty percent "displacement" (for calculation of cross reaction) is then equal to $B/T \times 100 = 75\%$, if $B_o/T \times 100 = 50\%$ (the "50% displacement" is half way between maximum binding and total displacement).

The construction of these sigmoidal curves is always somewhat arbitrary, and the readings of the values for unknowns may be difficult. The automatic, computerized handling of data offers great advantages over the manual one. For automatic handling a linearization of the curve must be performed. Routine dose interpolation of the entire standard curve, or restriction to a small linear segment of the curve, cannot be recommended. A better approach is to linearize the sigmoidal curve by applying, for example, a logit transformation of $(B - B_o)/(T - B_o) \times 100$ versus log dose (or $\log_e$ dose) (Rodbard et al., 1969).

For the sake of simplicity, $(B - B_o)/(T - B_o) \times 100$ is designated Y:

$$\text{logit}\,(Y) = \log_e\left[\frac{Y}{100 - Y}\right]$$

Thus: $Y = 50\%$ gives logit $(Y) = 0$
$Y = 90\%$ gives logit $(Y) = +2.2$
$Y = 10\%$ gives logit $(Y) = -2.2$

There is a linear relation between logit (Y) and log dose:

$$\text{logit}\,(Y) = a + k \cdot \log X$$

X — amount of unlabeled prostaglandin in a standard tube
k = slope of the line
a = intercept with the ordinate

The linear relationship between logit (Y) and log X may be plotted on semilogarithmic paper, after which manual calculations of a and k can be made. For determination of log X for the unknowns, the equation

$$\log X = \frac{\text{logit}\,(Y) - a}{k}$$

is used. The determination of a and k, however, preferably is not made manually from the plot; instead, unweighted or weighted least-square analyses should be performed.

We have chosen to perform this calculation with a Digital 81 Computer, which has the capacity of making a weighted least-square regression analysis of logit (Y) versus, in our case, $\log_e X$. Thus the values for a and k are found, the logit values from the unknown samples can be calculated, and finally the prostaglandin concentrations are determined. Further advantages of using this kind of computer is that it can also easily be programmed with, for instance, a dilution factor or a known content of protein in the unknown samples. The computer then gives the answers directly in picograms per milliter or per milligram of protein. Finally, special problems may arise, e.g., in the case of using two RIAs for the measurement of TXB_2 and mono-O-methyl TXB_2 in the same samples (Granström et al., 1976*b*) or for determining PGE_2 after reduction to $PGF_{2\alpha}$ and $PGF_{2\beta}$ with $NaBH_4$ (Lindgren et al., 1974). The prostaglandin or thromboxane values obtained from the two assays must be corrected for mutual cross reactions, which involves two problems: First, the cross reaction varies between different levels of the standard curve; and second, iterative counting must be performed for proper correction of cross reaction. Such cross reaction calculations are impossible to perform manually. It is necessary to use a computer for correct and reliable measurements.

Some scientists prefer to measure the radioactivity of the antibody-bound fraction instead, e.g., when dextran-coated charcoal is employed for the separation step or when the pellet is analyzed after a precipitation (PEG, second antibody, etc.). An analogous calculation procedure can be used in these cases. The radioactivity in the "maximum binding" tubes (B_o, no unlabeled compound present) minus the radioactivity found in the absence of antibody is set to 100%. (The radioactivity found in the absence of antibody (N) is caused by "nonspecific binding" or more often by incomplete removal of the free fraction, e.g., by omitting the reprecipitation of the pellet in the PEG procedure). The parameter $(B - N)/B_o - N) \times 100$ decreases with increasing log dose. Either a sigmoidal curve or a linear logit plot may be constructed also in this case.

If unextracted samples are analyzed in the RIA, it is extremely important to measure the nonspecific binding (N) both in the standard samples containing no antibody and in unextracted samples containing no antibody, or in unextracted samples containing antibody and a massive dose of the unlabeled compound (a recommended amount is 50 times the amount that gives $(B/B_o = 0.5)$ (Rodbard et al., 1969).

Our own analyses of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ in unextracted plasma indicated that nonspecific binding was negligible even when volumes as large as 0.5 ml plasma were assayed. The very few cpm found after reprecipitation in the "bound fraction" in the absence of antibody were less than 0.5% of the total radioactivity. Nevertheless this factor N must be carefully measured and studied in each specific problem, as it may be different with other compounds and under other circumstances.

EVALUATION OF THE RADIOIMMUNOASSAY

Even including the raising of the antiserum and preparation of the labeled ligand, standards, and other reagents necessary for

the method, the development of an RIA is neither particularly time-consuming nor difficult. Instead, the most tedious, difficult, and important step in the whole procedure is the subsequent evaluation of the assay. The remainder of this chapter deals with this particularly important aspect.

If RIA results are to provide reliable, unbiased measurements of prostaglandin concentrations, estimates of the errors in these determinations must be given together with data on the binding properties of the antibody, as well as the principles for the determination of assay reliability and methods for appropriate, efficient analysis of data.

Four generally accepted assay reliability criteria must be described with each assay: specificity, sensitivity, precision, and accuracy.

Specificity

Anything that competes with the proper antigen for the antibody binding sites, or in any other way affects the binding of labeled antigen, influences the final results. The antigen–antibody binding may be affected by nonspecific factors (nonimmunological inhibition) such as pH, osmolality, certain proteins and cations and by immunological competition by structurally related compounds. Specificity may be defined as the extent of freedom from interference by substances other than the one intended to be measured (Midgley et al., 1969). When estimating the specificity of an antiserum there is unfortunately a strong tendency to overlook the former, nonimmunological type of interference and measure only the cross reactions of other prostaglandins and related compounds with the antiserum.

The nonspecific interfering factors are discussed in more detail below. Suffice it to say here that the possible influence of these factors must be constantly kept in mind, that their effects may be difficult to detect, and that they may lead to highly erroneous results.

Cross reactions with structurally related compounds are often given in percents. These percentages are calculated from the amounts of the related compounds required to give a 50% displacement of the bound, labeled antigen from the binding sites compared to the amount of the unlabeled antigen required for the same displacement (= "100% cross reaction" for the proper antigen).

The cross reactivity of the antibody is often illustrated as a set of standard curves. The curves are the results of experiments where the antibody has been incubated with a constant amount of the radiolabeled, proper antigen, and attempts to inhibit the achieved binding have been made by adding unlabeled, related compounds in increasing amounts. Figures 6 and 8 show the cross reactivities of two antisera. The compound which has the highest affinity for the antibody should ideally be the compound for which the antibody was developed: Only minor amounts of unlabeled molecules are required to start the displacement of the labeled ones. This curve is thus found to the far left in the diagram. The lower affinity a compound has for the antibody, the larger amounts of it are needed to displace the radiolabeled molecules of the proper compound; thus these curves are found to the right in the diagram. Both figures clearly show that the more structurally related a substance is to the compound for which the antiserum was developed, the higher is the cross reactivity, *viz.*, the closer are the displacement curves.

The cross reaction in percent, defined as above, represents only the cross reaction at 50% displacement and does not give a true picture of the events over the total range of the standard curve. Thus this figure cannot be used for accurate calculations if the final values have to be corrected for the presence of a related prostaglandin. This can be done only if the displacement curves are absolutely parallel for two compounds, which occurs only rarely. If calculations have to be performed (Lindgren et al., 1974, Granström et al., 1976*b*), the cross reaction must be known at every point of the standard curve.

Cross reactions <0.1% can be considered to be without significance in most biological systems and do not have to be determined more accurately than this.

When studying the specificity of a certain antiserum, it is necessary to test the cross reactivity with the appropriate compounds, i.e., compounds known to occur in significant concentrations in the biological system to be studied. Thus when examining the properties of, for example, an antibody against a tetranor dioic metabolite of $PGF_{2\alpha}$ occurring in urine (Fig. 2), it is not correct to evaluate only its cross reactions with a number of primary prostaglandins or other C_{20} compounds which do not occur in urine at all or only in negligible quantities. The antibody will then exhibit a seemingly high degree of specificity, which does not give a true picture of its properties (cf. Fig. 8). No doubt many antisera against this compound cross react extensively with other tetranor and even dinor metabolites that occur in high concentrations in urine (Granström and Samuelsson, 1971*b*; Granström, 1972).

Sensitivity

Sensitivity is unfortunately defined differently by investigators. There are two principal definitions of sensitivity. The one found in all statistical books describes sensitivity in terms of the slope of the dose-response curve, i.e., the change in the response for a given change in the dose. The other definition is the detection limit of the assay system, i.e., the smallest amount of the unlabeled substance that can be distinguished from the presence of no substance with acceptable precision. Many scientists working with RIA do not distinguish between these two concepts and so use the term randomly in both senses. However, it seems that "the limit of detection" is what is most commonly meant by the word "sensitivity" in the field of prostaglandin RIA. For practical purposes it is convenient to use the amount of substance causing a 10% displacement from the "maximum binding" value as the limit of detection. It is essential, however, that the variation around this point is significantly different from the variation around the "zero" point (point of "maximum binding").

The sensitivity is influenced by a number of factors. First, it depends predominantly on the avidity of the antibody. (The terms "avidity" and "affinity" are often used as synonyms, and both describe the energy of binding exhibited by a particular antigen–antibody reaction. However, the term avidity actually refers only to the properties of the antibody, and affinity to those of the antigen.) The term "avidity" is essentially the same as the association constant in physical chemistry (*Ka*), with:

$$Ka = \frac{[\text{AbPG}]}{[\text{Ab}][\text{PG}]}$$

where [AbPG], [Ab], and [PG] refer to the molar concentrations of the antibody–prostaglandin complex, free antibody, and free prostaglandin, respectively. The avidity of the antibody can be determined by plotting the standard curve as a Scatchard plot (Scatchard, 1949). The ratio of antibody-bound to free antigen is plotted against the concentration of bound antigen (expressed as moles/liter); the avidity is then given by the slope of this line, and the absolute amount of antibody by its intercept on the abscissa.

The *Ka* values for prostaglandin antisera are seldom published. A few antisera for $PGF_{2\alpha}$ raised in rabbits have had *Ka* values ranging from 5 to 6×10^6 M^{-1}.

Many other factors also influence the sensitivity of the assay. The concentration of antibody should generally be kept low (Fig. 10). Excess of antibody results in loss of sensitivity, since all binding sites must be occupied before the unlabeled material being assayed can compete with and displace the labeled molecules.

The amount of the added labeled ligand influences the sensitivity in two ways. First, it must be kept in mind that a certain amount of substance is added to the tubes with this preparation, as the labeled molecules themselves and sometimes also as unlabeled molecules which may be present in a tracer of low spe-

cific activity, e.g., [9β-^{3}H]$PGF_{2\alpha}$. In most of the prostaglandin RIAs published to date, this amount ranges from approximately 4 pg (4,000 dpm of a ^{3}H-tracer with a specific activity of 150 Ci/mmole) to 300 pg (20,000 dpm, specific activity 10 Ci/mmole). This amount, which is inevitably added to the tubes, should be kept as low as possible by using a tracer with high specific activity. It cannot be expected that in a tube which already contains several hundred picograms of prostaglandin, an appreciable displacement of radioactivity shall take place if only an additional 5 pg is added.

On the other hand, the measured amount of radioactivity must be high enough to ensure reasonable counting reliability. Low radioactivity of the samples may of course be compensated for by longer counting times, but this may not be practically possible for routine use of RIA. As is the case with all radioactivity counting, the amount of radioactivity in the tubes is also important for the precision of the determinations.

As mentioned above, iodinated tracers provide an excellent alternative to the tritium-labeled ones, as they can be prepared with a considerably higher specific activity (Maclouf et al., 1976; Sors et al., 1977).

Precision and Accuracy

Precision and accuracy can be defined as follows: Precision is the extent to which a given set of measurements of the same sample agrees with the mean for that set. Accuracy is the extent to which the mean of an infinite number of measurements of a

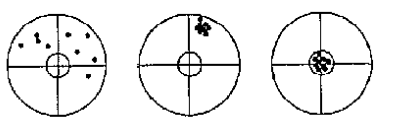

FIG. 11. The meaning of precision and accuracy illustrated as shots at a target.

substance agrees with the exact amount of the substance present (Midgley et al., 1969) (Fig. 11).

PRECISION

The precision is actually not influenced by the properties of the antiserum, the tracer, or any other reagent, but is a function of the skill of the scientist, the quality of the equipment, and so on. Two terms are employed in this connection: the within-assay (intraassay) variation and the between-assay (interassay) variation. The within-assay variation is obviously caused by minor unintentional variations in the handling of the samples at different stages of the procedure: pipetting, mixing, decanting, etc. This factor must be evaluated and given for each assay; a convenient way of presenting the data is suggested in Table 1. The intraassay variation can be expressed either as ± the standard deviation (SD, in absolute amounts; e.g., picograms) or, preferably, as ± the coefficient of variation (CV, in percent: SD/mean × 100). This variation must be estimated for different amounts of the substance, as it is not uniform over the range of the standard curve; it can be seen from Table 1 that it is higher in the lowest and highest regions. This is conveniently estimated by the simultaneous assay of many aliquots from several sample pools with different concentrations of the compound. In spite of the fact that this variation is not a property

TABLE 1. *Precision of measurements of 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid by RIA*

Range (pg)	Mean (pg)	Coefficient of variation (%)	No. of duplicate determinations
2– 30	18.9	12.3	74
31– 60	43.6	10.3	65
61– 90	73.7	6.8	34
91–120	105.0	7.1	38
121–150	135.8	9.3	31

inherent in the assay as such, but rather resides in the quality of pipettes and so on, it is important that these figures are given, otherwise it is impossible for the reader to evaluate the reliability of presented data.

The between-assay variation also depends on how well the assay is standardized. Deviation from the mean of a pool may be caused by variations in temperature, incubation time, sequence of additions, etc., but it is also influenced by the fact that samples and reagents may not be stable on storage.

ACCURACY

Estimating the accuracy is the most difficult problem when evaluating assay reliability. Ideally, this would be carried out by assay of biological samples with exactly known concentrations of prostaglandins. Such samples do not exist, however. Therefore several approaches are employed. A common experiment is the addition of known amounts of prostaglandin to plasma or some other biological fluid, followed by RIA; this should give a straight line. Figure 12 shows a typical result, and the value obtained from the sample to which no such addition was made is interpreted as the amount of prostaglandin already present in the biological material. (This is not necessarily the case, however; see below.) It is not legitimate to add the substance to buffer only, as the assay may behave in a quite different way in biological material or extracts thereof. Furthermore, the additions should be made in the low physiological range, where the assay is intended to be used. Unfortunately it is often seen that in this addition experiment scientists work with amounts many times higher than those that can be expected in biological material; they thus assay highly diluted aliquots, and so the effects of nonspecific interfering factors are not likely to be demonstrated. The addition experiment is a minimum requirement in evaluating a RIA; however, a straight line does not imply that the RIA is reliable, as this kind of experiment reveals only the presence of major disturbing factors.

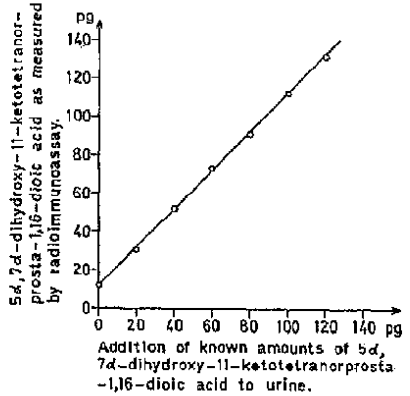

FIG. 12. Accuracy of an RIA for 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid determined by assay of urine to which increasing amounts of the compound was added.

The next experiment in the evaluation of accuracy is the most important one: comparison of RIA data with those obtained from a completely independent method (e.g., a mass spectrometric method or bioassay). It is not sufficient to compare data obtained from two RIAs, even though some information may be gained about nonspecific interferences in an assay by using two or more completely different precipitation methods, as was suggested above. If the laboratory does not have facilities for setting up two independent quantitative methods (e.g., RIA and mass spectrometry), it might be possible to collaborate with other laboratories in analyzing aliquots from the same sample pool by different methods. The mass spectrometric methods are by far the most specific and reliable ones; if, in comparison with such a method, the RIA scientist finds deviating values, the source of error is likely to reside in the RIA and must be sought (cf. Samuelsson, 1973). Sometimes the discrepancies are enormous. This is particularly the case with PGA compounds, for which a number of RIAs exist. This problem is discussed in more detail in the next section.

Other requirements that must be fulfilled by an accurate prostaglandin RIA is that it must clearly demonstrate increases or decreases in the prostaglandin levels under conditions which are known to be connected with such changes. A convenient experiment to test the validity of, for example, a RIA for 15-keto-13,14-dihydro-$PGF_{2\alpha}$ is to follow the plasma levels of this compound before, during, and after the administration of $PGF_{2\alpha}$ to a subject (Cornette et al., 1974; Kindahl et al., 1976*a*) or in connection with luteolysis in a suitable species (Fairclough and Payne, 1975; Peterson et al., 1975; Kindahl et al., 1976*a,b*). An RIA for urinary metabolites could be evaluated in the corresponding way (Granström and Kindahl, 1976*b*). It is likely, however, that even a RIA where nonspecific effects exert an influence will demonstrate superimposed increases under such conditions, and no conclusions can be drawn about the reliability of the "basal" levels detected with the method. [Compare, for example, the luteolysis studies in the bovine species published by Peterson et al. (1975) and Kindahl et al. (1976*a,b*); the "basal" plasma metabolite levels were around 250 pg/ml in the report by the first-mentioned authors, but around 60 to 80 pg/ml in the papers by Kindahl et al. However, the pulsatile release pattern seen during luteolysis was similar in the studies.]

The second type of experiment, aimed at demonstrating decreases in the prostaglandin levels, may give some information about the reliability of the "basal" levels. The prostaglandin synthetase inhibitors (e.g., indomethacin and aspirin) offer excellent possibilities for this type of study and have frequently been used *in vitro* and *in vivo* (e.g., Granström and Kindahl, 1976*a,b*; Granström et al., 1976*a*). Scientists sometimes report the inhibition of a certain prostaglandin *release* by indomethacin, for example, whereas the drug "failed to suppress the *basal* prostaglandin formation." The latter observation could hold true for *in vivo* experiments, where many pharmacological factors come into play, and the reason thus may be simply that a sufficient blood concentration of the inhibitor never was reached (caused by low absorption from the gastrointestinal tract, high

protein binding in plasma, rapid breakdown or excretion of the drug, etc.) (Fig. 13). For *in vitro* experiments, and particularly studies with a somewhat purified prostaglandin synthetase, failure of the drug to affect the "basal prostaglandin levels" should automatically lead to the suspicion that these so-called basal levels merely reflect a nonspecific effect on the antigen–antibody binding in the assay. This explanation is far more likely than that the cyclooxygenase should be resistant to the action of the inhibitor.

The importance of a proper investigation of the assay accuracy prompted us to devote the whole last section of this chapter to this problem. The fact that nonspecific influences on RIA results are frequently completely overlooked in the field of prostaglandin measurements (although they are well known in steroid, pro-

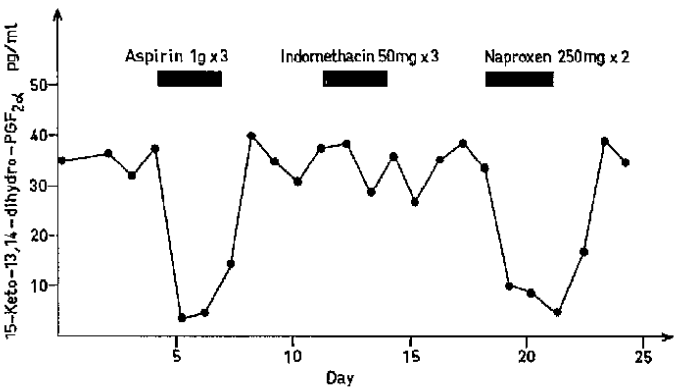

FIG. 13. Effects on prostaglandin production by aspirin, indomethacin, and naproxen in a human female. Blood plasma samples were collected daily for 24 days, and the prostaglandin synthetase inhibitors were administered orally during 3-day periods as indicated. The plasma samples were assayed for 15-keto-13,14-dihydro $PGF_{2\alpha}$. The study was carried out as a test for reliability of the assay. Since a remarkable decrease in the prostaglandin metabolite level was seen with aspirin and naproxen, the lack of decrease during the indomethacin treatment cannot be caused by inability of the assay to detect lowered levels of the compound. The phenomenon is likely to have a pharmacological explanation, i.e., sufficient plasma levels of indomethacin were probably never reached in this subject. None of the three drugs have been found to interfere in this RIA.

tein, and peptide hormone assays) further motivated a detailed discussion of this aspect.

CAN RADIOIMMUNOASSAY RESULTS BE TRUSTED?

Since the method was introduced into the field of prostaglandin measurement in 1970 to 1971, an immense amount of RIA data has been published. The reported levels from biological fluids or tissues have varied enormously, and so have the conclusions drawn from the data. Only a few examples of this confusion are mentioned here.

Gutierrez-Cernosek et al. (1972) reported that the serum $PGF_{2\alpha}$ level reached a peak during the second trimester of human pregnancy, with the highest value around 849 ± 429 pg/ml. In contrast, Brummer (1973) found that the $PGF_{2\alpha}$ levels were lowest during this period (390 ± 470 pg/ml) and were significantly higher during the first and last trimesters. Most investigators have found a rise during labor and delivery [e.g., Caldwell et al. (1971) (peripheral plasma level 1,200 to 5,000 pg/ml)]; however, Hennam et al. (1974) found plasma levels around 20 to 30 pg/ml throughout pregnancy with the lowest values during the second trimester and the rise during labor not exceeding the level during the first trimester. Twomey et al. (1975) found serum $PGF_{2\alpha}$ levels ranging from 0 to 2,000 pg/ml throughout pregnancy; no differences were seen between the different trimesters.

However, using a mass spectrometric method for determining the major urinary metabolite of $PGF_{1\alpha}$ and $PGF_{2\alpha}$, Hamberg (1974) demonstrated that there is a gradual increase in production of PGF throughout pregnancy. The maximum excretion value, corresponding to a four- to fivefold increase in the individual prepregnancy value, was found at the end of pregnancy.

The peripheral "$PGF_{2\alpha}$ levels" from nonpregnant subjects have also varied, with a tendency to decrease in later publications. In several reports from 1972, the values are in the nanogram range but decrease to 100 to 400 pg/ml in 1973 (e.g., Jaffe et al., 1973; Van Orden and Farley, 1973; Zusman et al., 1973) to 20 to 100 pg/ml in 1974 (Hennam et al., 1974; Youssefnejadian et al., 1974), and finally to approximately 10 pg/ml in 1975 (Dray et al., 1975). However, several laboratories still report $PGF_{2\alpha}$ levels in the nanogram range.

Prostaglandins of the "1" series are quantitatively of minor importance compared to those of the "2" series. Many scientists have published data on both types from peripheral serum or plasma [(both of which are likely to reflect the amounts formed during blood sampling (Samuelsson et al., 1975)]. Ritzi and Stylos (1974) found 10 times more PGE_2 than PGE_1 in human serum; Levine (1973) in analyses of F compounds found only $PGF_{2\alpha}$ and no detectable $PGF_{1\alpha}$. In contrast, Dray et al. (1975) found 10 times more PGE_1 and Van Orden et al. (1977) 2 to 3 times more PGE_1 than PGE_2 in human plasma.

Several reports have been published on the prostaglandin content of synovial fluid in rheumatoid arthritis. Robinson and Levine (1974) could not detect any PGE at all in such samples but found high PGB levels; Robinson and Granda (1974), on the other hand, found high amounts of PGE compounds.

The problem with PGA measurements was touched on in the introductory chapter. As mentioned there, PGAs are believed by some to be circulating hormones and among other things have been proposed to be involved in renal physiology and pathology (Lee, 1973*a*). "Peripheral plasma levels" of PGA compounds (generally $PGA_1 + PGA_2$) in the human have been published by many laboratories utilizing RIA (Table 2). Most of these levels are in the range of 1,000 pg/ml plasma and sometimes considerably higher. However, using recently developed mass spectrometric methods for PGA_2, the measured levels in human plasma were not significantly different from 0 (Table 3): the limit of detection in one method was 200 pg/ml (Frölich et al., 1975), and in the second method 5 pg/ml (Gréen and Steffenrud, 1976). That these enormous discrepancies should be caused by the presence of large amounts of PGA_1 in the blood is not likely. Besides, the only RIA that selectively measured

TABLE 2. *"Normal" levels of PGA/B compounds in the peripheral circulation assayed by RIA*

Reference	Level (pg/ml) Male	Level (pg/ml) Female (average)	Compound(s) assayed	Fluid
Jubiz et al. (1972)	40–770	120–810	PGA + B	Plasma
Zusman et al. (1972)	1,390 ± 550	1,620 ± 520 (1,150 ± 400)	PGA_1 + PGA_2	Plasma
Jaffe et al. (1973)	1,024 ± 214	880 ± 160 (1,062 ± 107)	PGA_1 + PGA_2	Plasma
Lee (1973b)		(1,200–2,400)	"PGA"	Plasma
Zusman et al. (1973)		(1,600 ± 60)	PGA_1 + PGA_2	Plasma
Bellivreau and Bachur (1974)		(3,270 ± 270)	PGA + E + B	Plasma
Pletka and Hickler (1974)	1,450 ± 90	1,500 ± 10	PGA_1 + PGA_2	Plasma
Van Orden et al. (1975)		1,286 ± 65	"PGA"	Plasma
Zia et al. (1975)	25 ± 16	18 ± 18	PGA_1	Whole blood
Zia et al. (1975)	19 ± 13		PGA_2	Plasma
Hornych et al. (1976)	130 ± 25	101 ± 18	PGA + B	Plasma

TABLE 3. *Basal levels of PGA_2 in human peripheral plasma as assayed by mass spectrometry*

Reference	Level (pg/ml) Male (average)	Level (pg/ml) Female (average)	Limit of detection (pg/ml)
Frölich et al. (1975)	(56 ± 134) (15 ± 166)		200
Gréen and Steffenrud (1976)	<9	<10	5

PGA_1 gave the lowest levels (approximately 20 pg/ml) (Zia et al., 1975). Nor could the extremely high RIA levels be explained by cross reactions with other prostaglandins, as quite unphysiological levels of these generally would be required to give such results.

It is thus no wonder that the critical reader gets the impression that with the aid of RIA it is possible to obtain almost any kind of data and prove almost anything. There is a definite tendency—although with many exceptions—for RIA levels to decrease in later reports. This seems to occur roughly parallel to the development of mass spectrometric methods for the compound in question. For "peripheral plasma levels" of primary prostaglandins, the most striking decrease occurred during 1973 after the development of mass spectrometric assays for PGE_2 and $PGF_{2\alpha}$. For the 15-keto-13,14-dihydro metabolites, the mass spectrometric methods already existed and plasma data were published before most scientists took an interest in these compounds; using the RIA methods later developed for these metabolites, low levels were nearly always reported (Granström and Samuelsson, 1972; Stylos et al., 1973; Fairclough and Payne, 1975; Granström and Kindahl, 1976*a;* Sors et al., 1977). The same correlation can be seen for the assays for urinary metabolites: mass spectrometric methods had provided data on the basal excretion of the dioic acids already by 1972 and 1973 (Hamberg, 1972, 1973), whereas the first RIAs were not developed until 1974 to 1976 (Ohki et al., 1974, 1975, 1976; Cornette et al., 1975; Granström and Kindahl, 1976*a,b*). The values

obtained using these RIAs have agreed well with the mass spectrometric data published earlier. On the other hand, in an area like the PGA analyses, where mass spectrometric methods were not developed until lately (1975 and 1976), the RIA levels have remained high until recently (Table 2).

Thus one often gets the impression that the reported RIA values of prostaglandins often do not reflect biological events but merely the state of development of mass spectrometry.

In most reviews on assay methodology, where RIA is compared to other quantitative methods, one finds the phrase "RIA is a very specific method." This is, however, far from true in many cases. What can be stated with certainty is that the *antibody* often has a very high degree of specificity, but this does not necessarily mean that the final results are reliable. It should be pointed out that in all the above-mentioned studies, where highly diverging results were obtained, the assays have always fulfilled the common reliability criteria fairly well. The antisera employed have been of somewhat variable quality, particularly regarding their cross reactions with related prostaglandins. Most have been of a reasonably good quality, however; and from the published properties of the assays, no obvious source of error can generally be found. The large discrepancies can be explained only by the unfortunately very common misinterpretation of RIA results. It must be stressed that the inhibition of the antigen–antibody binding is by no means always caused by the substance being measured or even by related substances; the inhibition may be mainly nonimmunological.

After finishing a particular assay, the scientist obtains information about how much radioactivity is found in, for instance, the free fraction. After quench correction, this figure is translated into picograms of prostaglandin using the standard curve from the same experiment. It is important to realize that the RIA does not actually allow this procedure. The amount of the radiolabeled molecules found may exist in the free fraction because they have been properly displaced from the antibody binding sites by the unlabeled molecules of the same compound. This is the

ideal situation, and then a high "free" radioactivity is correctly interpreted as a large amount of the measured prostaglandin. The free radiolabeled molecules may also have been displaced from the binding sites by a related compound or "other immunoreactive material" which occurred in high concentration in the sample—an example of a common cross reaction between a not completely specific antibody and other compounds. Most scientists are aware of this risk and carefully report their results not as picograms of the particular prostaglandins but as picograms of "prostaglandin-like material." If the antibody is definitely nonselective (e.g., some PGE antiserum that cross reacts extensively with PGA or PGB compounds), this care is almost always taken, and the final results are reported as "PGE equivalents" or using some similar expression.

However, if the cross reactivity curves show that the antibody is of a high specificity and does not cross react significantly with any other compound that can be expected to occur in the samples, there is often a strong tendency to believe in the final results. Thus a multitude of factors are neglected, factors that interfere with the antigen–antibody binding in a completely nonspecific way. It is important to remember that the standards and the samples can never be assayed under absolutely identical conditions. Thus any factor present in the samples—a salt, protein, or compound belonging to other classes of substances—that somehow prevents the antigen–antibody binding from taking place can also produce as a final result a comparatively high radioactivity count in the free fraction. This is then erroneously interpreted as if a large amount of unlabeled prostaglandin had been present in the sample.

The majority of these factors are of course unidentified. However, sometimes it has been possible to identify the nature of such a nonspecific effect and perhaps to eliminate its influence:

pH: Too high or too low pH is known to inhibit the antigen–antibody binding. For prostaglandins an acidic pH may occur in the assay after an extraction step, as acidification of the samples is a prerequisite for the extraction of prostaglandins into an or-

ganic solvent. If the extracts are not carefully washed until neutral afterward, the pH in the sample tubes of the RIA may become low enough to interfere with the antigen–antibody binding. This difficulty may of course be overcome by using a more concentrated buffer in the assay, but then the next nonspecific factor may come into play.

Ionic strength: High ionic strength also inhibits the antigen–antibody binding. Buffers more concentrated than 0.01 to 0.05 M are generally not recommended (*Standardization . . .*, 1974). When analyzing unextracted samples of a fluid that normally has a variable composition (e.g., urine), this factor is particularly important to keep in mind; and if the assay is not sensitive enough to allow considerable dilution of the samples, the final results must be viewed with some suspicion.

Heparin: This compound is often used as an anticoagulant and is a well-known inhibitor of antigen–antibody binding in many RIAs. It is probably safe to use if care is taken that the concentration of heparin does not exceed the minimum amount necessary to prevent clotting, i.e., 10 IU/ml.

Factors influencing immunoprecipitation: Factors likely to be of a protein nature in plasma from several species are known to inhibit especially the binding of a second antibody to the first antigen–antibody complex (cf., e.g., Utiger et al., 1962; Buckler, 1971; Court and Hurn, 1971; and references in these papers). Variations in salt and protein concentration also influence the efficiency of the immunoprecipitation. Inhibition of this second step in the assay leads to the same result as mentioned above. Incomplete precipitation of the antigen–antibody complex means that the properly bound but unprecipitated label will be found in the free fraction, and again the final result will be interpreted as a "high prostaglandin value." A survey of the literature in this field shows that prostaglandin levels obtained using the double-antibody technique generally are considerably higher than those obtained in assays with other separation techniques.

Several of the above-mentioned factors may be removed by an extraction step, but this does not automatically lead to higher

reliability for values of extracted samples. First, there is the possibility that the extraction procedure concentrates an unwanted interfering substance in the sample along with the desired prostaglandin, even substances that give rise to a false parallelism in the dilution test performed after the extraction. It has also been found that compounds of lipidic nature, which are likely to be concentrated during a prostaglandin extraction, can inhibit an antigen–antibody reaction, e.g., in a RIA for plasma oxytocin (Chard, 1971).

Second, the extraction step may itself introduce interfering substances, e.g., solvent impurities, impurities from the gas used for evaporation, leakage from chromatographic columns. In our experience, even extremely purified solvents generally gave some blank reaction in the assay; a small volume of distilled water carried through the procedure almost always proved to contain some 5 to 15 pg of "prostaglandin" in the later performed assay. This constituted a problem, since the physiological levels of several prostaglandin metabolites are in the same order of magnitude and since it is not legitimate merely to subtract a blank value from measurements of unknowns.

As mentioned above, we found evaporation of the samples to be the most deleterious step in a common extraction procedure. The best solution to the problem was to let the solvent evaporate spontaneously to dryness at room temperature. Evaporation under a stream of N_2 invariably increased the procedure blank value several-fold, up to as much as 25 to 50 pg of "prostaglandin" per sample. This occurred even after extensive washing of the gas. That the interfering substance(s) introduced during this step was not of prostaglandin nature (and thus the phenomenon was not a common cross reaction) is shown by the fact that evaporation with N_2 never disturbs the gas chromatographic—mass spectrometric measurements of many prostaglandins and related compounds in our laboratory.

This holds true also for the next nonspecific factor we encountered. During one stage of our attempts to find a good extraction method, we tried microcolumns of Amberlite XAD-2

for the extraction of urinary or plasma samples. Unfortunately, this extremely efficient, reproducible, rapid method proved to be impossible to use in connection with RIA because the column constantly leaked polymer, which almost completely inhibited the antigen–antibody binding. Even the most extensive washing of the column prior to use did not eliminate this leakage. Neither in this case is the inhibition of the antigen–antibody binding caused by prostaglandin-like material, since no such material was ever detected using mass spectrometric methods. In fact, Amberlite XAD-2 is part of the routine of the gas chromatographic–mass spectrometric determination of prostaglandins in our laboratory. The presence of this interfering substance eluted from XAD-2 must have been detected by many scientists. It is a striking fact that this otherwise superior extraction method is almost never employed in connection with RIA. There is, however, one recent report where XAD-2 was used for extraction of prostaglandins from incubation media (Laychock and Rubin, 1976). These authors found a fivefold increase in their "prostaglandin levels" when the resin was employed compared to a common extraction procedure. No attempts were made to explain this phenomenon. Fretland (1974) investigated the extraction properties of several ion-exchange resins with the aim of finding a suitable extraction procedure for RIA purposes. Unfortunately, this investigator never tried the dried eluates in any RIA, only the unextracted samples; otherwise, it is likely that the same deleterious influence would have been detected.

Thus, as mentioned above, extraction of samples for a RIA may introduce more problems than it solves; however, the use of unextracted samples is also obviously connected with the risk of the presence of interfering substances. The choice between extraction and no extraction is really a choice between Scylla and Charybdis, and no universal solution can be provided to ensure completely reliable results at the end of the assay.

The best thing the scientist can do is *to be aware of the possibility* that there are interfering factors present in his samples, and that a certain or even a major part of the inhibition of the

antibody–tracer binding during the incubation may be caused by these factors.

The opposite problem also exists, *viz.*, enhancement of the antigen–antibody binding by synergistically acting factors. This is not the same phenomenon as nonspecific binding, which may be exerted by albumin or other compounds since no binding by the synergistically acting factors occurs in the absence of antibody. This synergism is neither as common nor as well studied as the inhibitory factors discussed above but has nevertheless been encountered (Kirkham and Hunter, 1971). We have sometimes seen it in unextracted horse plasma and occasionally in human synovial fluid. The synergism is a serious problem, is difficult to detect, and leads to completely misleading results. The presence of this phenomenon may be revealed by values that fall below zero, i.e., a binding of tracer exceeding that in the maximal binding tube or by the lack of parallelism in the dilution tests. Even if exceedingly low values are not found in an assay, one cannot safely conclude that no synergistically acting factors occurred in the samples, as their influence might have been counteracted by one or more of the inhibiting factors discussed above.

If synergism is found, one can sometimes destroy these influencing factors by repeated freezing and thawing of the samples. However, such an approach is generally not recommended. Instead, extraction of the samples may solve the problem.

There are a few possibilities of demonstrating the presence of nonspecificity in a RIA, some of which were briefly mentioned in the previous section. The first possibility is, as mentioned, to compare the data with those obtained using a different method applied to the same samples, preferably a mass spectrometric assay. If this is not possible, one may at least get some information by comparing one's data with published data from similar studies (*not* data obtained using RIAs!), with the specific question in mind: Are these data physiologically possible? Such a critical comparison would have eliminated a large number of publications on, for example, "primary prostaglandin levels" in

peripheral circulation. The "physiological plasma levels" of PGE and F compounds have often been in the order of magnitude of 1 to 2 ng/ml in RIA publications and occasionally even very much higher. It is well known that a $PGF_{2\alpha}$ level of 2 to 5 ng/ml plasma is sufficiently high to induce abortion, and even a level of 500 pg to 2 ng/ml is connected with frequent side effects, e.g., uterine cramps, vomiting, and diarrhea. These values have repeatedly been found from mass spectrometric measurements of peripheral plasma levels in women receiving $PGF_{2\alpha}$ for therapeutic abortion (Beguin et al., 1972; Granström et al., 1973; Gréen, 1973; Green et al., 1972).

These suggestions—comparative studies using different methods, or comparison with data in the literature—give information only about the possible presence of nonspecific, interfering factors in the RIA and do not solve the problem of how to make the RIA valid. The modifications that may be necessary include: the use of a more avid antiserum; elimination of an extraction step or, on the contrary, introduction of such a step or a change in the extraction method; use of a different precipitation method, etc. If these suggested approaches do not give the desired improvement, it is still possible to make the assay somewhat more reliable; the design of the study should be made in such a way that *single* samples are avoided. The assay of single samples—obtained from one subject on one occasion—means that the scientist has to report an absolute level of the compound in question. This is largely impossible using RIA, as has been amply illustrated by the many examples given above. Instead of measuring absolute levels, the scientist should aim at following *changes* in the prostaglandin concentration.

Our own studies are almost always designed in this way. A large number of samples are collected, with short intervals, from the same subject or the same reaction vessel during a time when a change in the prostaglandin or thromboxane level can be expected. The collection starts well in advance of the expected release (or inhibition) and is continued for a sufficiently long period afterward to allow reliable estimations of the "basal

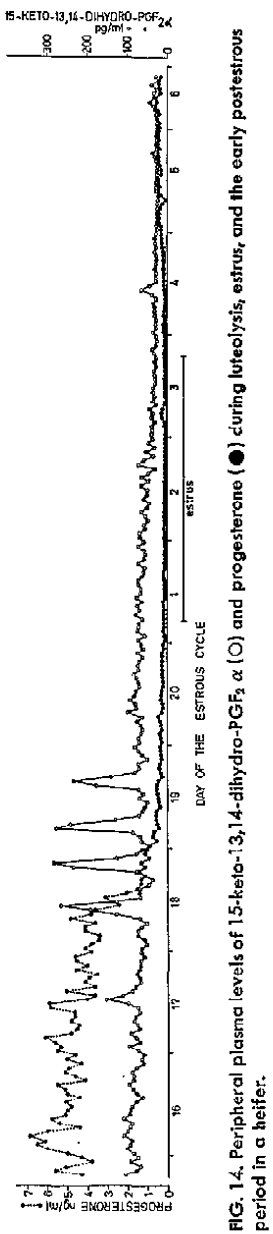

FIG. 14. Peripheral plasma levels of 15-keto-13,14-dihydro-$PGF_2\alpha$ (○) and progesterone (●) during luteolysis, estrus, and the early postestrous period in a heifer.

levels." This kind of experimental design is illustrated in Figs. 13 through 17. Figure 13 shows the inhibition of prostaglandin production in a human female, as reflected by the peripheral plasma levels of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ before, during, and after the oral ingestion of aspirin, indomethacin, and naproxen. (The lack of prostaglandin decrease seen during indomethacin administration was commented on above and is likely to have a pharmacodynamic explanation.)

Figure 14 shows the release of $PGF_{2\alpha}$ as reflected by peripheral plasma levels of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ during normal luteolysis in a heifer. A fairly even "basal level" of this compound (50 to 80 pg/ml) was interrupted by several frequent high peaks of short duration concomitant with the decrease in progesterone 3 to 4 days prior to estrus (Kindahl et al., 1976*b*).

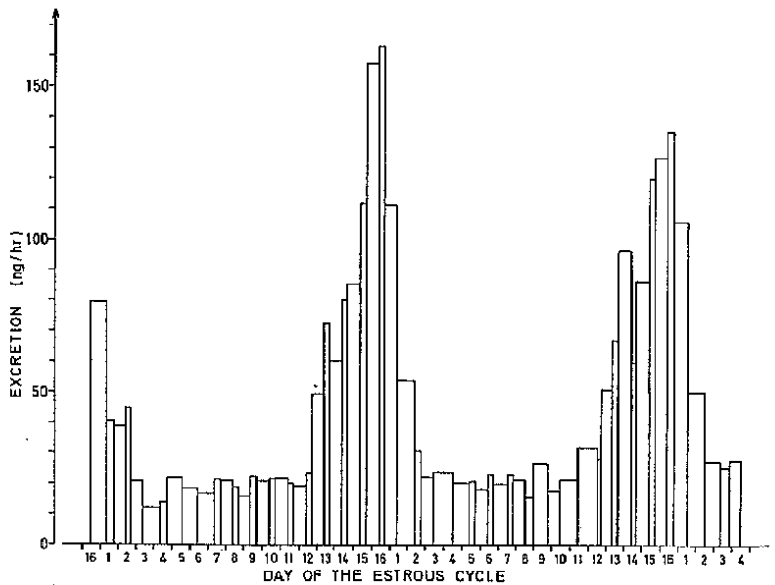

FIG. 15. Excretion of 5α,7α-dihydroxy-11-ketotetranorprostanoic acid into urine of a guinea pig during two consecutive estrous cycles. Day 1 of the cycle = day of heat.

Figure 15 shows a similar study in the guinea pig. This time the urinary excretion of the main $PGF_{2\alpha}$ metabolite was monitored, *viz.*, 5α,7α-dihydroxy-11-ketotetranorprostanoic acid. Low, constant excretion of the metabolite was seen during the major part of the cycle; on day 12 the prostaglandin production started to increase and reached a peak the day before estrus (Granström and Kindahl, 1976*b*).

Figure 16 demonstrates a study on thromboxane production during platelet aggregation, as reflected by the increasing levels of the stable TXB_2. The aggregation was induced by collagen addition to platelet-rich plasma; when indomethacin was in-

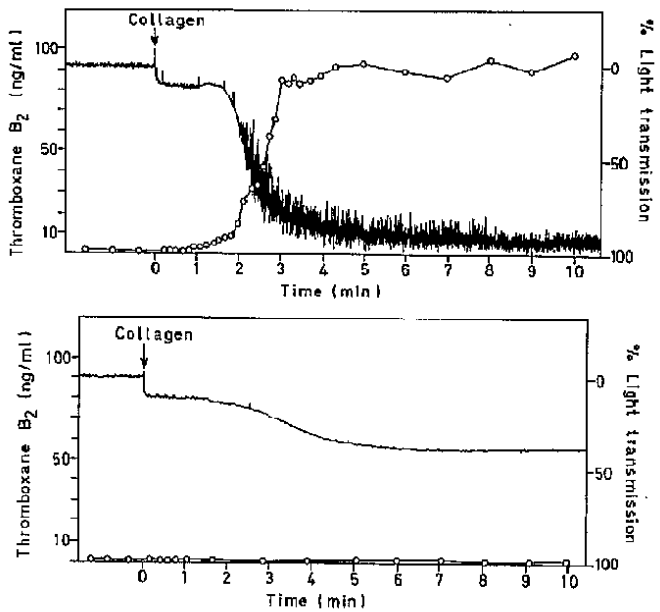

FIG. 16. Release of TXB_2 (○) after addition of collagen to human platelet-rich plasma (PRP). **Upper panel:** PRP without indomethacin. **Lower panel:** PRP containing 1.4×10^{-4} M indomethacin. Light transmission was recorded simultaneously in an aggregometer.

cluded in the incubation mixture, no aggregation took place, and no thromboxane formation occurred (Granström et al., 1976*a*).

Figure 17 demonstrates a similar study where arachidonic acid was added to a suspension of washed human platelets. In this experiment the formation of the unstable TXA_2 was monitored after conversion to the stable mono-O-methyl TXB_2 by excess methanol. The two compounds, TXB_2 and mono-O-methyl TXB_2, were measured using separate RIAs. After correction for mutual cross reactions of the compounds in the two assays, the half-life of the unstable TXA_2 in aqueous medium could be accurately calculated.

It must be stressed that in neither of these examples can we

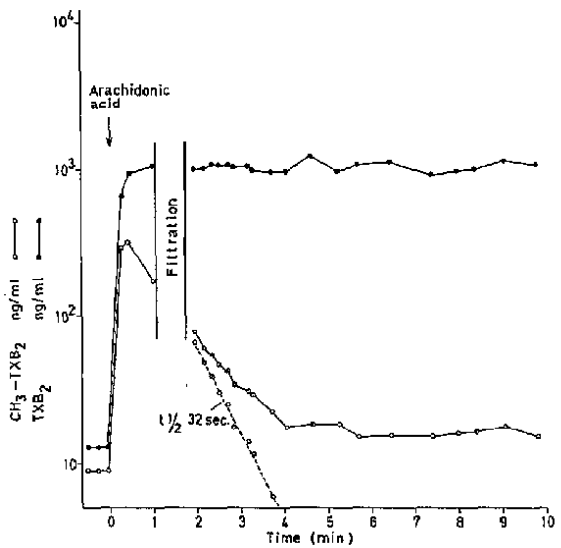

FIG. 17. Mono-O-methyl-TXB_2 and TXB_2 levels after addition of arachidonic acid to washed human platelets at 37°C. Samples were withdrawn before and after the platelets had been removed by filtration. (O——O) CH_3-TXB_2 uncorrected for cross reaction with TXB_2. (O- - -O) CH_3-TXB_2 corrected for cross reaction with TXB_2. (●——●) TXB_2 corrected for cross reaction with CH_3-TXB_2.

be absolutely certain of the accuracy of the "basal levels." Comparative studies with mass spectrometric methods have been performed in most cases, and the levels have been found to agree well. The accuracy of the individual sample, however, is always uncertain. Using this kind of experimental design, the importance of the individual "basal" value is minimized and the study as a whole acquires a high degree of certainty. We do not advocate analysis of single or very few samples, e.g., the type of study that was frequently performed earlier; one sample from the first, second, and third trimester from pregnant females, etc. Even with a large number of subjects, the information provided by such analyses is minimal. It is far more valid to perform studies in a limited number of subjects with a large number of samples from each subject. Prostaglandin production (e.g., during pregnancy) is best studied by the continuous analysis of urinary metabolites (Hamberg, 1973; Granström and Kindahl, 1976*b*).

The possible role of prostaglandins in various pathological conditions is gaining increasing interest. In many kinds of clinical studies, the "serial" experimental design may be impossible, e.g., when studying a chronic disease without distinct fluctuations in the clinical picture. The comparison of plasma or urinary levels of prostaglandins in these patients with those of healthy subjects is unfortunately utterly hazardous using RIA for the many reasons given in this section. Reported increases or decreases in pathological conditions must always be viewed with some skepticism unless the actual change in prostaglandin production was really caught with a series of frequent samples.

DEVELOPMENT OF A RIA: SUMMARY

1. Decide on what organ or other biological material to study and select the proper compound for this study from the knowledge of precursor acid metabolism. Develop the RIA for this compound or a stable derivative thereof.

2. Whichever sample type is assayed (extracted or unextracted), the possibility of the presence of nonspecific interfering

factors must be constantly kept in mind. Make dilution tests and assay for parallelism; however, do not accept parallelism as an absolute criterion of absence of nonspecific influence.

3. Use two or more completely different separation techniques for comparison when validating the RIA.

4. When evaluating the RIA, do not limit the investigation to the traditional reliability criteria: sensitivity, specificity, precison, and accuracy. The RIA may seem reliable at this stage and still give completely unrealistic values when applied to biological material. The method must be evaluated by other studies as well.

5. In the experimental design, aim at following the changes in prostaglandin levels in a series of samples instead of measuring absolute levels in single samples.

6. Make comparative studies employing different quantitative methods.

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Bioassay of Prostaglandins and Biologically Active Substances Derived from Arachidonic Acid

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A series of new and sophisticated chemical methods, including gas chromatography, radioimmunoassay, and mass spectrometry, are being developed and perfected for detection and quantification of prostaglandins (PGs) and related substances. One should not forget, however, that starting with the discovery of prostaglandins, biological techniques and bioassay have contributed more than any other method to the development of the field. Bioassay has provided crucial information on the role of lungs in the removal of circulating prostaglandins (1), the participation of prostaglandins in inflammatory reaction (2,3), the contribution of prostaglandins to the autoregulation and maintenance of blood flow to the kidney (4–6), the inhibitory effect of aspirin-like drugs on the biosynthesis of prostaglandins (7–9), the mediation of pyrogen fever by prostaglandins (10), and the release of rabbit aorta-contracting substance (RCS) from lungs during anaphylaxis (11) (now identified as thromboxane A_2) (12); moreover, in 1976 it made possible the discovery of PGX, the latest member of the prostaglandin family (13–16), now

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